c-*met* siRNA ADENOVIRUS VECTORS INHIBIT CANCER CELL GROWTH, INVASION AND TUMORIGENICITY

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention in the field of molecular biology and medicine relates to interfering RNA (RNAi) compositions and methods of using them to reduce the expression of the Met oncogene, a receptor for hepatocyte growth factor/scatter factor (HGF/SF), in tumor cells. This promotes apoptosis and results in inhibition of tumor cell growth, invasion and metastasis.

Description of the Background Art

Hepatocyte growth factor/scatter factor (HGF/SF) is a pleiotropic factor that induces a wide range of cellular functions, including proliferation, migration, angiogenesis and morphogenesis (Gherardi, E *et al.*, *Cancer Cells 3:*227-232, 1991; Takebayashi, T *et al.*, *J Cell Biol 129:*1411-1419, 1995; Jeffers, M *et al., Cell Growth Differ 7:*1805-1813, 1996; Sonnenberg, E *et al., J Cell Biol 123:*223-235, 1993) Met, the only known receptor for the HGF/SF ligand, can mediate signaling to downstream molecules (Birchmeier, C *et al. Trends Cell Biol 8:*404-410, 1998; Stewart, F *Rev Reprod 1:*144-148, 1996; Furge, KA *et al., Oncogene 19:*5582-5589, 2000; Birchmeier, C *et al., Nat Rev Mol Cell Biol 4:*915-925, 2003; Bottaro, D *et al., Science 251:*802-804, 1991).

HGF/SF is produced mainly by mesenchymal cells, while Met is preferentially expressed in epithelial and endothelial cells (Jeffers, M *et al.*, Oncogene *13:*853-856, 1996; Yang, XM *et al., Lab Invest 73:*483-491, 1995; Sonnenberg, E *et al., Exs 65:*381-394, 1993). In many types of tumor cells, Met signaling is activated through ligand-dependent autocrine or paracrine mechanisms (Park, WS *et al., Apmis 108:*195-200, 2000; Morello, S *et seq., J Cell Physiol 189:*285-290, 2001). Enhanced signal transduction via the stimulation of this receptor contributes to the malignant phenotype. Activating mutations in the Met receptor, first discovered in human papillary renal carcinomas (Schmidt, L *et al., Nat Genet 16:*68-73, 1997) have now been discovered in several different types of cancers and metastatic lesions. In mouse models, these mutations induce transformation, proliferation and invasion *in vitro*, as well as tumorigenicity and metastasis *in vivo* (Jeffers, M *et al., Oncogene 17:*2691-2700, 1998; Jeffers, M *et al., Proc Natl Acad Sci U S A 94:*11445-11450, 1997)

HGF/SF binding to Met activates signaling downstream (Ponzetto, C *et al., Cell 77:*261-271, 1994) through various pathways such as the Ras mitogen-activated protein kinase (MAPK) pathways through Grb2-SOS complex formation (Ponzetto, C *et al., J Biol Chem 271:*14119-14123, 1996) or the Ras and Rac pathways (Ridley, AJ *et al., Mol Cell Biol 15:*1110-1122, 1995) responsible for tubulo-morphogenesis (Sachs, M *et al., J Cell Biol 133:*1095-1107, 1996) and cell spreading/actin

reorganization. Likewise, the STAT pathway, particularly STAT3 activation, is required for HGF/SF-Met–mediated growth in soft agar (Zhang, YW *et al., Oncogene 21:*217-226, 2002), and PI3 kinase activation followed by Akt phosphorylation contributes to the prevention of apoptotic cell death (Xiao, GH *et al., Proc Natl Acad Sci U S A 98:*247-252, 2001; Fan, S *et al., Mol Cell Biol 21:*4968-4984, 2001). In addition, HGF/SF-Met signaling can up-regulate the production of matrix metalloproteinases and urokinase that induce the degradation of extracellular matrices and basement membrane and enhance tumor invasion and metastasis (Harvey, P *et al., Br J Cancer 83:*1147-1153, 2000; Kermorgant, S *et al., Carcinogenesis 22:*1035-1042, 2001). Moreover, the activation of Met protein (Jeffers *et al.*, 1997, *supra*) is involved in the induction of blood vessel formation in tumors by increasing the production of VEGF (Rosen, EM *et al., Ciba Found Symp 212:*215-226, 227-229, 1997; Tomita, N *et al., Circulation 107:*1411-1417, 2003) and by simultaneously shutting off the anti-angiogenesis factor thrombospondin-1 (Zhang, YW *et al., Proc Natl Acad Sci USA in press* 2003; U.S.S.N 60/484,676).

Since HGF/SF-Met signaling is implicated in a wide range of tumors and regulates biological activities that contribute to the tumor cell malignancy, targeting the Met receptor has become a subject of interest in the field of cancer biology (Birchmeier *et al., supra*). Cao *et al.* (Cao, B *et al., Proc Natl Acad Sci U S A 98:*7443-7448, 2001) showed that HGF/SF-neutralizing antibodies efficiently block *in vitro* scattering and branching morphogenesis and can suppress the *in vivo* growth of HGF/SF-Met signal–dependent glioblastoma cell xenografts in nude mice. This suggests that interrupting the autocrine and/or paracrine HGF/SF-Met signaling in tumors that depend on this pathway is a potential intervention strategy.

The administration of c-*met*-antisense oligonucleotides is another molecular approach used to block Met function in cancer cells. A recent report showed that (a) c-*met*-antisense oligonucleotides decreased Met protein levels in the LoVo human colon cancer cell line and (b) apoptotic cell death (induced by serum deprivation) was more prominent in antisense-treated cells than in controls (Kitamura, S *et al., Br J Cancer 83:*668-673, 2000).

Production of the dominant negative ("DN") forms of the Met protein is another approach to suppressing Met function. DN-Met in DA3 mouse mammary adenocarcinoma cells reduces tumorigenicity *in vivo* and metastatic potential (Firon, M *et al., Oncogene 19:*2386-2397, 2000). By using DN- Met that has inactivating mutations at both ATP binding sites in the kinase domain and at two important Tyr residues in the multidocking site, Furge *et al.* showed that inhibition of the Met receptor can suppress Ras-mediated metastasis (Furge, KA *et al., Proc Natl Acad Sci U S A 98:*10722-10727, 2001). Ribozymes that target Met mRNA constitute a more direct approach to suppressing Met expression. Abounader *et al.* designed a hammerhead ribozyme against Met and showed that reduction of Met expression by the ribozyme suppressed anchorage-independent *in vitro* colony formation and *in*

vivo tumorigenicity (Abounader, R *et al., J Natl Cancer Inst 91:*1548-1556, 1999. These investigators also showed that Met targeting by the ribozyme suppressed tumor growth and angiogenesis and, in turn, promoted apoptotic cell death (Abounader, R *et al., Faseb J 16:*108-110, 2002). Christensen *et al.* disclosed that selective inhibition of Met tyrosine kinase activity using ATP-competitive small molecules had anti-tumor effects and thus anti-cancer therapeutic potential (Christensen, JG *et al., Canc Res 63:*7345-7355, 2003).

RNA interference (RNAi) is a recently reported phenomenon that has developed into a new approach for elucidating gene function. RNAi is a sequence-specific, post-transcriptional, gene-silencing mechanism that is effected through double-stranded RNA (dsRNA) molecules homologous to a sequence of the target gene (Elbashir, SM *et al., Nature 411:*494-498, 2001; Fire, A *et al., Nature 391:*806-811, 1998; Tuschl, T *et al., Genes Dev 13:*3191-3197, 1999). Fragments of the dsRNA called "small interfering" RNAs (siRNAs) can rapidly induce loss of function, and only a few molecules are required in a cell to produce the effect (Fire *et al., supra*) through hybrid formation between a homologous siRNA and mRNA (Lin, SL *et al., Curr Cancer Drug Targets 1:*241-247, 2001). A member of the RNase III family of nucleases named *dicer* has been identified as being involved in processing (Bernstein, E *et al., Nature 409:*363-366, 2001). DNA vector–mediated RNAi technology has made it possible to develop therapeutic applications for use in mammalian cells (Sui, G *et al., Proc Natl Acad Sci USA 99:*5515-5520, 2002; McCaffrey, AP *et al., Nature 418:*38-39, 2002; Lee, NS *et al., Nat Biotechnol 20:*500-505, 2002). There have been several reports of delivery by retroviral vectors for stable expression (Barton, G.M *et al., Proc Natl Acad Sci USA 99:*14943-14945, 2002; Paddison, PJ *et al., Cancer Cell 2:*17-23, 2002; Rubinson, DA *et al., Nat Genet 33:*401-406, 2003; Tiscornia, G *et al., Proc Natl Acad Sci USA 100:*1844-1848, 2003) or adenoviral vectors for transient expression (Xia, H *et al., Nat Biotechnol 20:*1006-1010, 2002).

Citation of the above documents is not intended as an admission that any of the foregoing is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

SUMMARY OF THE INVENTION

The present inventors produced adenovirus vectors encoding siRNA sequences directed against both mouse and human Met and under the control of the U6 promoter. RNAi encoded by these constructs effectively silenced *met* RNA and protein expression in all cell types examined. The abrogation of Met strongly inhibited *in vitro* cell proliferation, scattering, and migration, all of which are outcomes of HGF/SF-stimulation via the Met receptor. More importantly, Met abrogation also induced

apoptosis and suppressed tumor development and growth *in vivo*. Thus c-*met* siRNA vectors are useful as for targeting and treating Met expressing cancers. Since *c-met* is involved in the process of proliferation, invasion and metastasis in a vast range of tumor types, the present adenoviruses and other vectors carrying *c-met* siRNA may be directed against a particularly broad range of cancers characterized by activation of the Met signalling pathway.

The present invention is directed to a interfering RNA (RNAi) molecule having a sequence that is sufficiently complementary to the sequence of mRNA encoded by human c-*met* (SEQ ID NO:1) or murine c-*met* (SEQ ID NO:2) so that expression of the RNAi molecule in a cell that normally expresses c-*met* results in diminution or loss of expression of the mRNA. The RNAi molecule may be a single stranded siRNA that forms a hairpin structure or a double stranded siRNA.

It should be understood that when a nucleotide sequence is written herein with bases that include thymine (T), a characeristic of DNA, the "identical" RNA sequence contains a uracil base (U) at that position, or if a DNA coding sequence is shown, , the encoded RNA sequence will have a U at a position corresponding to (complementary to) an adenine (A) of the coding sequence.

Preferably, the above RNAi molecule comprises, or, in the alternative, consists essentially of, a sequences selected from the group consisting of SEQ ID NO:9; SEQ ID NO:10; SEQ ID NO:11; SEQ ID NO:12; SEQ ID NO:13; SEQ ID NO:14; and SEQ ID NO:15, or a sequence that is complementary to one of said sequences of this group..

Also provided is a DNA molecule encoding any of the above RNAi molecules.

Another embodiment provides an expression construct comprising DNA encoding the above RNAi molecule, operatively linked to a promoter that drives the expression of the RNAi in a c-*met*-expressing cell. The promoter is preferably one that drives the expression of the RNAi in a c-*met*-expressing tumor or cancer cell, such as a polIII promoter, a preferred example of which is a U6 promoter.

The invention is directed to a vector, preferably a viral vector, comprising the above expression construct. This vector can be a transient or a stable expression vector. Preferred are adenoviral vectors, in particular an Ad5 viral vector. Preferred Ad5 vectors include those encoding a human, murine or canine Met-directed siRNA: (a) si-mMet-Ad5^{57}; (b) si-mMet-Ad5^{60}; (c) si-mMet-Ad5^{110}; (d) si-mMet-Ad5^{178}; (e) si-hMet-Ad5^{16}; (f) si-hMet-Ad5^{62}; (g) si-hMet-Ad5^{221} (h) si-dMet-Ad5^{111}; (i) si-dMet-Ad5^{197}; and (j) si dMet-Ad5 ^{222}.

Another embodiment of the invention is a method for inhibiting c-*met* expression in a c-*met* expressing cell, comprising modifying the cell so that it expresses (a) the above RNAi molecule, (b) the above DNA molecule, or (c) the above expression construct, under conditions effective to inhibit the c-*met* expression. Another method comprises infecting the cell with the above viral vector under

conditions that are effective for (i) expression of the RNAi molecule, and thereby (ii) inhibition for c-*met* expression. Preferably c-*met* expression is inhibited for at least 3 days after expression of the RNAi.

The cell is preferably a tumor or cancer cell, most preferably a human cell.

In the above method, the inhibiting of c-*met* expression reduces the ability of the cell to bind and respond to stimulation by HGF/SF.

In the above method, the RNAi molecule may be expressed in the cell *in vitro* or preferably, *in vivo*. Preferably, the cell and the expression vector reside in a subject with cancer.

Also provided is a method for inhibiting proliferation, invasion and/or metastasis of a c-*met*$^+$ tumor cell or killing the tumor cell, comprising modifying the cell so that it expresses the above RNAi molecule, DNA molecule, or expression construct, thereby inhibiting the proliferation, invasion and/or metastasis or killing the tumor cell. The method may also comprise infecting the cell with the above viral vector in a manner effective for expression of the RNAi molecule, and inhibition for c-*met* expression, thereby inhibiting the proliferation, invasion and/or metastasis or killing the tumor cell, typically by apoptosis. In this method, the RNAi molecule may be expressed *in vitro* or, preferably, *in vivo*, such as in tumor cells in a subject with cancer.

The cancer that may be targeted by the above compositions and methods include several categories, as set forth in Table 1, namely, a carcinoma, a musculoskeletal sarcoma, a soft tissue sarcoma, a hematopoietic malignancy, or another cancer type (*e.g.*, glioblastoma, astrocytomas, melanoma, mesothelioma and Wilms' tumor).

The invention includes a method of treating a c-*met*$^+$ tumor or cancer in a subject, comprising administering to the subject an amount of the above viral vector effective for inhibiting expression of c-*met* and thereby (i) inhibiting the growth, invasion or metastasis of cells of the tumor or cancer, or (ii) killing the tumor or cancer cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D show effect of c-*met* siRNA adenovirus infection on Met expression in tumor cells. **Fig. 1A:** M114 mouse *met*-transformed NIH3T3 cells were infected with four different c-*met* siRNA adenoviruses at multiplicity of infection ("moi") of 10, 50, and 100. After three days, cells were harvested and Met expression was determined by Western blot (20 μg protein per lane). Met expression was suppressed by si-mMet-Ad5^{57} and si-mMet-Ad^{178} at moi=50 and 100. No significant reduction in Met expression was observed in mock virus that has only the mU6 promoter. The strongest RNAi effect was from si-mMet-Ad5^{178}. **Fig. 1B:** DBTRG human glioblastoma cells were infected with three different constructs of c-*met* siRNA adenoviruses at different moi (0, 10, 50, 100). After 3 d., cells were harvested and Met expression was determined as above. Met expression was dramatically suppressed by

si-hMet-Ad5 numbers 16, 62, and 221 at moi's from 10-100. si-hMet-Ad5^{221} showed the strongest RNAi effect. No reduction in Met expression was observed with mock mU6-Ad5 virus. **Fig. 1C**: Left panel: PC-3 human prostate cancer cells were infected with si-hMet-Ad5 viruses at moi=100. After three days, cells were harvested and the Met expression determined (30 μg protein/lane). Again si-hMet-Ad5^{221} showed the strongest RNAi effect. A mixture of all three vectors (si-hMet-Ad5$^{16, 62, 221}$) at moi=33.3 each gave a similar effect. Right panel: PC-3 cells were infected with mock (mU6-Ad5) or c-*met* siRNA (si-hMet Ad5^{221}) adenoviruses at different moi (0, 10, 50, 100). Cells were harvested and Met expression determined as above (30 μg protein/lane). Met expression was suppressed by si-hMet-Ad5^{221} dose dependently. **Fig. 1D:** MKN45 human gastric cancer cells were infected with mock (mU6-Ad5) or c-*met* siRNA (si-hMet-Ad5^{221}) adenoviruses at different moi of 0, 10, 50, and 100. Cells were harvested and Met expression determined as above (30 μg protein/lane). This cell line expresses a high level of Met. Again Met expression was dramatically suppressed by si-hMet-Ad5^{221} at moi=50-100.

Figures 2A-2B show effects of Met siRNA adenovirus on cell morphology. **Fig. 2A** shows morphological changes in M114 cells after infection with *c-met* siRNA adenovirus. Top: M114 cells were infected with mU6-Ad5, si-mMet-Ad5^{110}, or si-mMet-Ad5^{178} adenovirus at moi=100 and cultured for 5 d. Cells infected with si-mMet Ad5 were less refractile and more adherent (arrows). Non-infected control or mock virus–infected M114 cells remained refractile in appearance (200x magnification). Middle: M114 cells were infected with si-mMet-Ad5^{178} adenovirus at different moi (0, 10, 50, and 100). After three days, cell growth was suppressed dose dependently. Again cells infected with si-mMet Ad5^{178} were less refractile and more adherent (100x magnification). Bottom: To reduce the effect of cell density, si-mMet-Ad5^{178}-infected M114 cells were trypsinized at day 4 after infection, and each sample reseeded in a 6-well culture plate at the same cell density of (10^5 cells/well). Cells were observed 24 hr later. Less refractile change was dominant at moi=50-100 (100x magnification). **Fig. 2B**: DBTRG cells were infected with si-hMet-Ad5^{221} adenovirus at moi's of 10, 50, 100. After four days, cell appearance was observed. Non-infected control cells grew well and displayed a palisade pattern. In contrast, cells infected with si-mMet-Ad5^{221} were less spindle-shaped and revealed a wider cytoplasm. At the same time many cells rounded up and lost contact with the surface at moi=100 (100x magnification).

Figures 3A-3B. Fig 3A shows specificity of the si-Met-Ad5 adenoviruses. DA3 mouse mammary adenocarcinoma cells were infected with mock virus and si-mMet or si-hMet-Ad5 viruses at moi=100. Met expression (day 3) was strongly suppressed only in the cells infected with si-mMet-Ad5^{178}; no reduction in Met protein was observed in cells infected with the most potent RNAi for human Met, si-hMet-Ad5^{221}. **Fig. 3B:** Analysis of *met* mRNA expression by RT-PCR. Total RNA was extracted from the DA3 cells infected with mock or si-mMet-Ad5^{178} viruses at moi=100 for 3 days. Expression of *met* mRNA was dramatically reduced only in si-mMet-Ad5^{178}-infected cells.**Figures 4A**

and 4B. **Fig. 4A** DA3 cells were seeded at low cell density and cultured for 24 h and stimulated with 100 ng/ml HGF/SF for 48 h. Cell scattering was observed under a phase contrast. The DA3 scattered after treatment with HGF/SF (100x magnification). **Fig. 4B:** RNAi prevented HGF/SF-induced cell scattering. DA3 cells were infected with mU6-Ad5 or si-mMet-Ad5 57, 110, or 178 at moi=100 and cultured for 3 days. After reseeding, cells were cultured in low-serum medium for 24 h and treated with 100 ng/ml HGF/SF for 24 h. Cell scattering activity was suppressed in cells infected with the Ad5 viruses containing *met* siRNA coding sequences relative to mock infected cells (100x magnification).

Figures 5A-5C show RNAi persistence after cell passage. **Fig. 5A:** M114 cells were infected with si-mMet-Ad5^{178} at moi=100. Three days after infection, cells were trypsinized and reseeded (passage 1). This was repeated after three more days (passage 2). RNAi persisted even through passage 2. **Fig. 5B:** SK-LMS-1 cells were infected with si-hMet-Ad5^{6}2 at moi=100 and Met expression was followed up to the second passage as described for M114 cells. No remarkable reduction in Met expression was seen 3 d after infection, but significant suppression was observed after passage 1 and through passage 2. **Fig. 5C:** Time course of Met reduction by c-*met* siRNA adenovirus. DA3 cells were infected with si-mMet-Ad5 viruses at moi=100. Cells were harvested after 24, 48, or 72 h following infection, and Met expression level was determined by Western blot. Thirty micrograms of protein was loaded in each lane. The RNAi effect was observed with all si-mMet-Ad5 viruses from 24 h to 72 h. Adenovirus si-mMet-Ad5^{178} produced the strongest effect. A mixture, of all three (si-mMet-Ad5$^{57, 110, 178}$) was no better. This RNAi effect lasted after cell passage, but a reduction of cell viability was observed with si-mMet-Ad5-infected cells

Figure 6 shows results of a TUNEL assay of si-mMet-Ad5^{178} adenovirus-infected DA3 cells. The DA3 cells were infected with si-mMet-Ad5^{178} viruses at different moi (0, 10, 50, 100). Cells were fixed with formaldehyde at day 3 and 6, and apoptotic cells were detected by a TUNEL assay. Apoptotic cells are stained with dark brown (arrows). Three days after infection, suppression of cell growth is obvious at moi=50-100, and there are some apoptotic cells in si-mMet-Ad5^{178} adenovirus-infected cells. At day 6, many apoptotic cells are detected at moi=10-100.

Figure 7 shows quantitative analysis of apoptosis after infection with Met siRNA adenovirus. DA3, MKN45, DBTRG, and PC-3 cells were infected with si-Met-Ad5 viruses (si-mMet-Ad5^{178} for DA3 and si-hMet-Ad5^{221} for MKN45, DBTRG, and PC-3) at different moi's (0, 10, 50, 100). Three and six days after infection, both detached and adherent cells were collected and stained with propidium iodide (PI). The cells were analyzed by flow cytometry, and sub-G_1 fraction (*i.e.*, apoptotic fraction) was calculated. Three days after infection, the cells began to become apoptotic at moi=50-100. At day 6, dramatic increase in the apoptotic fraction was observed in DA3 and MKN45 cells.

Figure 8 shows that RNAi suppresses phosphorylation of Met and downstream signaling. **Fig 8A:** DA3 cells pretreated with the serum-free medium for 24 h were stimulated with HGF/SF (100 ng/ml). Cell lysates were collected and Met, ERK1,2 (p44/42 MAPK), and Akt phosphorylation were analyzed by Western blot. In response to HGF/SF, DA3 cells showed rapid phosphorylation of Akt and p44/42 MAPK. Although some phosphorylation of Met was observed before the HGF/SF stimulation, phosphorylation increased until 2 h, then decreased gradually. **Fig. 8B:** DA3 cells were infected with si-mMet-Ad5^{178} virus for 3 d. Cells were transferred to serum-free medium and cultured for 24 h. Cell lysates were collected 10 min after HGF/SF (100 ng/ml) treatment and protein phosphorylation was analyzed by Western blot. After HGF/SF treatment, control and mock virus-infected cells showed phosphorylation of Met, Akt, and MAPK. Phosphorylation of these molecules was marginal or inhibited in si-mMet-Ad5 virus-infected cells. There was no obvious change in the expression level of non-phosphorylated Akt in si-mMet-Ad5-infected cells.

Figures 9A-9B show effects of c-*met* siRNA on *in vivo* tumorigenicity of DA3 cells. DA3 cells were infected with si-mMet-Ad5^{178} virus *in vitro* at moi=100. After 3 d, cells were trypsinized and resuspended at 10^6/ml. BALB/c mice were inoculated subcutaneously (sc) in the right flank with 10^5 cells. Tumor size was observed and recorded twice a week. DA3 cells infected with si-mMet-Ad5^{178} did not form tumors for 21 days. Each symbol and bar represents the mean±SD of 10-11 animals. * $p<0.001$ compared to non-infected control and $p<0.01$ compared to mock (mU6-Ad5) infection. **Fig. 9B:** DA3 cells (10^5) were inoculated sc into the right flanks of BALB/c mice. Three and seven days later (indicated by arrows), 4 x 10^7 pfu of si-mMet-Ad5^{178} virus (in 0.1 ml PBS) was injected into the tumor lesion. Tumor size was followed and recorded for 24 days. Control: PBS only, Mock: mU6-Ad5 (4 x 10^7 pfu). Each symbol and bar represents the mean±SD of eight to ten animals. * $p<0.05$ compared to PBS control and $p<0.01$ compared to mock (mU6-Ad5) virus treatment. ** $p<0.01$ compared to both PBS control and mock virus treatment. There was no statistical significance between PBS control and mock virus-treated groups.

Figures 10A and 10B show effects of RNAi on MDCK nontransformed canine epithelial cells vs. TR6LM canine prostate carcinoma cells. Fig. 10 shows effects of dog Met siRNA adenovirus constructs on Met expression. MDCK and TRFl6M cells were infected with three different constructs of Ad Met SiRNA at different moi's (50 and 100). After 3 days, cells were harvested and met expression level was observed by Western blot. Thirty μg of protein was loaded in each lane. Fig. 10B is a series of micrographs showing effects of dMet-Ad5 adenoviruses on the scattering of MDCK cells. MDCK cells were infected with mU6-AD5 or si-dMet-Ad5$^{111, 197 \text{ or } 222}$ at moi=5 and cultured for 3 days. After reseeding, cells were cultured for 24 hrs and treated with 25 mg/ml HGF/SF for 24 hrs. Cell scattering activity was suppressed in a Met-dependent manner (100x magnification).

Figures 11A-11D show effectsof c-*met* siRNA adenovirus infection on Met expression in human ovarian cancer. The following three human ovarian carcinoma cell lines were used: CAOV3, ES2, SKOV-3 and OVMZ6 (Munich). Western blots are shown, as described for Figure 1A-D and in Example 1. Western blot was done on day 4 after infection with the human Met-directed constructm, si-hMet-Ad5^{221} (30 mg protein/lane) (Figs. 11A, B and D). SKOV-3 cells (Fig. 11C) were infected with the adenovirus while in suspension, and cells were then plated in culture dish.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present inventors designed vectors that express siRNA sequences that hybridize to, and block activation of c-Met, a protein tyrosine kinase receptor for HGF/SF. The present invention is directed to the siRNA molecules (sequences), vectors, preferably adenovirus vectors, with a promoter, exemplified by the U6 promoter, that drives transcription of siRNA sequences that are "specific" for sequences of *c-met* nucleic acid. siRNA "hairpin" sequences are preferred because of their stability and binding to the target mRNA.

Since *c-met* is involved in the process of proliferation, invasion and metastasis in a vast range of tumor types, the present adenoviruses carrying *c-met* siRNA may be directed against a particularly broad range of cancers characterized by activation of the Met signalling pathway. A nonlimiting list of such cancers appears in Table 1.

Table 1: List of Met-Expressing Cancers*

Category	Cancer Type	HGF/SF expression	Poor Prognosis	Mutation of Met
Carcinomas	Bladder	+	+	–
	Breast	+	+	–
	Cervical	+	+	–
	Colorectal	+	–	–
	Esophageal	–	–	–
	Gastric	+	+	+
	Head and Neck	+	+	+
	Kidney	+	–	+
	Liver	+	+	+
	Lung	+	+	+
	Nasopharyngeal	+	+	–
	Ovarian	–	–	+
	Pancreas/Gall Bladder	+	–	–
	Prostate	+	–	–
	Thyroid	+	+	–
Musculoskeletal sarcomas	Osteosarcoma	+	–	–
	Synovial Sarcoma	+	+	–
	Rhabdomyosarcoma	–	–	–
Soft tissue sarcomas	MFH/Fibrosarcoma	+	–	–
	Leiomyosarcoma	+	–	–
	Kaposi's Sarcoma	+	–	–
Hematopoietic Malignancies	Multiple Myeloma	+	+	–
	Lymphomas	+	–	–
	Adult T Cell Leukemia	–	–	–
Other Neoplasms	Glioblastomas/Astrocytomas	+	+	+
	Melanoma	+	–	–
	Mesothelioma	+	–	–
	Wilms' Tumor	+	–	–

* many of these express HGF/SF, indicating autocrine stimulation of the Met pathway.

For each cancer type, headings correspond to studies showing: HGF/SF expression in tumor biopsies; Met expression in tumor biopsies; expression of HGF/SF or Met correlating with poor prognosis; sporadic or germline-activating mutations in Met; tumor cells *in vitro* expressing Met or HGF/SF, some with correlation to *in vitro* neoplastic-like activities; and animal models supporting the role of Met and HGF/SF in cancer, including human tumor xenografts in immune-compromised mice, mice with HGF/SF or Met transgenes, or other animal models displaying dependence on HGF/SF or Met in cancer development. MFH=malignant fibrous histiocytoma;

+ indicates "Yes" for HGF/SF expression, poor prognosis and presence of Met mutation;

– indicates no report of HGF/SF expression, poor prognosis or presence of Met mutation

Human and Murine cMet

The human met gene nucleotide sequence (*hmet*) is shown below (SEQ ID NO:1) and has the Genbank accession number NM_000245. The coding sequence begins at nt 189 and continues up to nt 4415 (the last 5 nt's leading up to this point are boldfaced below. The signal peptide is encoded by nt's 189-260 and the met protooncogene product is encoded by nt's 261-4412. The HGF receptor α chain is encoded by nt's 261-1097 (underscored, all caps). The HGF receptor β chain is encoded by nt's 1110-4358 (underscored low case).

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   1 cgccctcgcc gcccgcggcg ccccgagcgc tttgtgagca gatgcggagc cgagtggagg
  61 gcgcgagcca gatgcggggc gacagctgac ttgctgagag gaggcgggga ggcgcggagc
 121 gcgcgtgtgg tccttgcgcc gctgacttct ccactggttc ctgggcaccg aaagataaac
 181 ctctcataat gaaggccccc gctgtgcttg cacctggcat cctcgtgctc ctgtttacct
 241 tggtgcagag gagcaatggg GAGTGTAAAG AGGCACTAGC AAAGTCCGAG ATGAATGTGA
 301 ATATGAAGTA TCAGCTTCCC AACTTCACCG CGGAAACACC CATCCAGAAT GTCATTCTAC
 361 ATGAGCATCA CATTTTCCTT GGTGCCACTA ACTACATTTA TGTTTTAAAT GAGGAAGACC
 421 TTCAGAAGGT TGCTGAGTAC AAGACTGGGC CTGTGCTGGA ACACCCAGAT TGTTTCCCAT
 481 GTCAGGACTG CAGCAGCAAA GCCAATTTAT CAGGAGGTGT TTGGAAAGAT AACATCAACA
 541 TGGCTCTAGT TGTCGACACC TACTATGATG ATCAACTCAT TAGCTGTGGC AGCGTCAACA
 601 GAGGGACCTG CCAGCGACAT GTCTTTCCCC ACAATCATAC TGCTGACATA CAGTCGGAGG
 661 TTCACTGCAT ATTCTCCCCA CAGATAGAAG AGCCCAGCCA GTGTCCTGAC TGTGTGGTGA
 721 GCGCCCTGGG AGCCAAAGTC CTTTCATCTG TAAAGGACCG GTTCATCAAC TTCTTTGTAG
 781 GCAATACCAT AAATTCTTCT TATTTCCCAG ATCATCCATT GCATTCGATA TCAGTGAGAA
 841 GGCTAAAGGA AACGAAAGAT GGTTTTATGT TTTTGACGGA CCAGTCCTAC ATTGATGTTT
 901 TACCTGAGTT CAGAGATTCT TACCCCATTA AGTATGTCCA TGCCTTTGAA AGCAACAATT
 961 TTATTTACTT CTTGACGGTC CAAAGGGAAA CTCTAGATGC TCAGACTTTT CACACAAGAA
1021 TAATCAGGTT CTGTTCCATA AACTCTGGAT TGCATTCCTA CATGGAAATG CCTCTGGAGT
1081 GTATTCTCAC AGAAAAGaga aaaaagagat ccacaaagaa ggaagtgttt aatatacttc
1141 aggctgcgta tgtcagcaag cctggggccc agcttgctag acaaatagga gccagcctga
1201 atgatgacat tcttttcggg gtgttcgcac aaagcaagcc agattctgcc gaaccaatgg
1261 atcgatctgc catgtgtgca ttccctatca aatatgtcaa cgacttcttc aacaagatcg
1321 tcaacaaaaa caatgtgaga tgtctccagc atttttacgg acccaatcat gagcactgct
1381 ttaataggac acttctgaga aattcatcag gctgtgaagc gcgccgtgat gaatatcgaa
1441 cagagtttac cacagctttg cagcgcgttg acttattcat gggtcaattc agcgaagtcc
1501 tcttaacatc tatatccacc ttcattaaag gagacctcac catagctaat cttgggacat
1561 cagagggtcg cttcatgcag gttgtggttt ctcgatcagg accatcaacc cctcatgtga
1621 attttctcct ggactcccat ccagtgtctc cagaagtgat tgtggagcat acattaaacc
1681 aaaatggcta cacactggtt atcactggga agaagatcac gaagatccca ttgaatggct
1741 tgggctgcag acatttccag tcctgcagtc aatgcctctc tgccccaccc tttgttcagt
1801 gtggctggtg ccacgacaaa tgtgtgcgat cggaggaatg cctgagcggg acatggactc
1861 aacagatctg tctgcctgca atctacaagg ttttcccaaa tagtgcaccc cttgaaggag
1921 ggacaaggct gaccatatgt ggctgggact ttggatttcg gaggaataat aaatttgatt
1981 taaagaaaac tagagttctc cttggaaatg agagctgcac cttgacttta agtgagagca
2041 cgatgaatac attgaaatgc acagttggtc ctgccatgaa taagcatttc aatatgtcca
2101 taattatttc aaatggccac gggacaacac aatacagtac attctcctat gtggatcctg
2161 taataacaag tatttcgccg aaatacggtc ctatggctgg tggcacttta cttacttaa
2221 ctggaaatta cctaaacagt gggaattcta gacacatttc aattggtgga aaaacatgta
2281 ctttaaaaag tgtgtcaaac agtattcttg aatgttatac cccagcccaa accatttcaa
2341 ctgagtttgc tgttaaattg aaaattgact tagccaaccg agagacaagc atcttcagtt
2401 accgtgaaga tcccattgtc tatgaaattc atccaaccaa atcttttatt agtacttggt
2461 ggaaagaacc tctcaacatt gtcagttttc tattttgctt tgccagtggt gggagcacaa
2521 taacaggtgt tgggaaaaac ctgaattcag ttagtgtccc gagaatggtc ataaatgtgc
2581 atgaagcagg aaggaacttt acagtggcat gtcaacatcg ctctaattca gagataatct
2641 gttgtaccac tccttccctg caacagctga atctgcaact ccccctgaaa accaaagcct
2701 ttttcatgtt agatgggatc ctttccaaat actttgatct catttatgta cataatcctg
2761 tgtttaagcc ttttgaaaag ccagtgatga tctcaatggg caatgaaaat gtactggaaa
2821 ttaagggaaa tgatattgac cctgaagcag ttaaaggtga agtgttaaaa gttggaaata
2881 agagctgtga gaatatacac ttacattctg aagccgtttt atgcacggtc cccaatgacc
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2941 tgctgaaatt gaacagcgag ctaaatatag agtggaagca agcaatttct tcaaccgtcc
3001 ttggaaaagt aatagttcaa ccagatcaga atttcacagg attgattgct ggtgttgtct
3061 caatatcaac agcactgtta ttactacttg ggtttttcct gtggctgaaa aagagaaagc
3121 aaattaaaga tctgggcagt gaattagttc gctacgatgc aagagtacac actcctcatt
3181 tggataggct tgtaagtgcc cgaagtgtaa gcccaactac agaaatggtt tcaaatgaat
3241 ctgtagacta ccgagctact tttccagaag atcagtttcc taattcatct cagaacggtt
3301 catgccgaca agtgcagtat cctctgacag acatgtcccc catcctaact agtggggact
3361 ctgatatatc cagtccatta ctgcaaaata ctgtccacat tgacctcagt gctctaaatc
3421 cagagctggt ccaggcagtg cagcatgtag tgattgggcc cagtagcctg attgtgcatt
3481 tcaatgaagt cataggaaga gggcattttg gttgtgtata tcatgggact ttgttggaca
3541 atgatggcaa gaaaattcac tgtgctgtga aatccttgaa cagaatcact gacataggag
3601 aagtttccca atttctgacc gagggaatca tcatgaaaga ttttagtcat cccaatgtcc
3661 tctcgctcct gggaatctgc ctgcgaagtg aagggtctcc gctggtggtc ctaccataca
3721 tgaaacatgg agatcttcga aatttcattc gaaatgagac tcataatcca actgtaaaag
3781 atcttattgg ctttggtctt caagtagcca aagcgatgaa atatcttgca agcaaaaagt
3841 ttgtccacag agacttggct gcaagaaact gtatgctgga tgaaaaattc acagtcaagg
3901 ttgctgattt tggtcttgcc agagacatgt atgataaaga atactatagt gtacacaaca
3961 aaacaggtgc aaagctgcca gtgaagtgga tggctttgga aagtctgcaa actcaaaagt
4021 ttaccaccaa gtcagatgtg tggtcctttg gcgtcgtcct ctgggagctg atgacaagag
4081 gagccccacc ttatcctgac gtaaacacct ttgatataac tgtttacttg ttgcaaggga
4141 gaagactcct acaacccgaa tactgcccag acccccttata tgaagtaatg ctaaaatgct
4201 ggcaccctaa agccgaaatg cgcccatcct tttctgaact ggtgtcccgg atatcagcga
4261 tcttctctac tttcattggg gagcactatg tccatgtgaa cgctacttat gtgaacgtaa
4321 aatgtgtcgc tccgtatcct tctctgttgt catcagaaga taacgctgat gatgaggtgg
4381 acacacgacc agcctccttc tgggagacat catagtgcta gtactatgtc aaagcaacag
4441 tccacacttt gtccaatggt tttttcactg cctgaccttt aaaaggccat cgatattctt
4501 tgctccttgc cataggactt gtattgttat ttaaattact ggattctaag gaatttctta
4561 tctgacagag catcagaacc agaggcttgg tcccacaggc cagggaccaa tgcgctgcag
```

The amino acid sequence of the human Met protein (1408 residues) is shown below (SEQ ID NO:2).

```
MKAPAVLAPG ILVLLFTLVQ RSNGECKEAL AKSEMNVNMK YQLPNFTAET PIQNVILHEH
HIFLGATNYI YVLNEEDLQK VAEYKTGPVL EHPDCFPCQD CSSKANLSGG VWKDNINMAL
VVDTYYDDQL ISCGSVNRGT CQRHVFPHNH TADIQSEVHC IFSPQIEEPS QCPDCVVSAL
GAKVLSSVKD RFINFFVGNT INSSYFPDHP LHSISVRRLK ETKDGFMFLT DQSYIDVLPE
FRDSYPIKYV HAFESNNFIY FLTVQRETLD AQTFHTRIIR FCSINSGLHS YMEMPLECIL
TEKRKKRSTK KEVFNILQAA YVSKPGAQLA RQIGASLNDD ILFGVFAQSK PDSAEPMDRS
AMCAFPIKYV NDFFNKIVNK NNVRCLQHFY GPNHEHCFNR TLLRNSSGCE ARRDEYRTEF
TTALQRVDLF MGQFSEVLLT SISTFIKGDL TIANLGTSEG RFMQVVVSRS GPSTPHVNFL
LDSHPVSPEV IVEHTLNQNG YTLVITGKKI TKIPLNGLGC RHFQSCSQCL SAPPFVQCGW
CHDKCVRSEE CLSGTWTQQI CLPAIYKVFP NSAPLEGGTR LTICGWDFGF RRNNKFDLKK
TRVLLGNESC TLTLSESTMN TLKCTVGPAM NKHFNMSIII SNGHGTTQYS TFSYVDPVIT
SISPKYGPMA GGTLLTLTGN YLNSGNSRHI SIGGKTCTLK SVSNSILECY TPAQTISTEF
AVKLKIDLAN RETSIFSYRE DPIVYEIHPT KSFISTWWKE PLNIVSFLFC FASGGSTITG
VGKNLNSVSV PRMVINVHEA GRNFTVACQH RSNSEIICCT TPSLQQLNLQ LPLKTKAFFM
LDGILSKYFD LIYVHNPVFK PFEKPVMISM GNENVLEIKG NDIDPEAVKG EVLKVGNKSC
ENIHLHSEAV LCTVPNDLLK LNSELNIEWK QAISSTVLGK VIVQPDQNFT GLIAGVVSIS
TALLLLLGFF LWLKKRKQIK DLGSELVRYD ARVHTPHLDR LVSARSVSPT TEMVSNESVD
YRATFPEDQF PNSSQNGSCR QVQYPLTDMS PILTSGDSDI SSPLLQNTVH IDLSALNPEL
VQAVQHVVIG PSSLIVHFNE VIGRGHFGCV YHGTLLDNDG KKIHCAVKSL NRITDIGEVS
QFLTEGIIMK DFSHPNVLSL LGICLRSEGS PLVVLPYMKH GDLRNFIRNE THNPTVKDLI
GFGLQVAKAM KYLASKKFVH RDLAARNCML DEKFTVKVAD FGLARDMYDK EYYSVHNKTG
AKLPVKWMAL ESLQTQKFTT KSDVWSFGVV LWELMTRGAP PYPDVNTFDI TVYLLQGRRL
LQPEYCPDPL YEVMLKCWHP KAEMRPSFSE LVSRISAIFS TFIGEHYVHV NATYVNVKCV
APYPSLLSSE DNADDEVDTR PASFWETS      1408
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The murine *c-met* gene nt sequence is shown below (SEQ ID NO:3). This is the coding sequence (total of 4841 nt's)

```
  1 atgaaggctc ccaccgtgct ggcacctggc attctggtgc tgctgttgtc cttggtgcag
 61 aggagccatg gggagtgcaa ggaggcccta gtgaagtctg agatgaacgt gaacatgaag
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 121 tatcagctcc ccaacttcac ggcagaaacc cccatccaga atgtcgtcct acacggccat
 181 catatttatc tcggagccac aaactacatt tatgttttaa atgacaaaga ccttcagaag
 241 gtatccgaat tcaagaccgg gcccgtgttg gaacacccag attgtttacc ttgtcgggac
 301 tgcagcagca aagccaattc atcaggaggg gtttggaaag acaacatcaa catggctctg
 361 cttgttgaca catactatga tgatcaactc attagctgtg gcagtgtcaa cagagggact
 421 tgccagcggc atgtccttcc tcctgacaat tctgctgaca tccagtctga ggtccactgc
 481 atgttctccc cagaagagga gtcagggcag tgtcctgact gtgtagtgag tgccctcgga
 541 gccaaagtcc tcctgtcgga aaaggaccgg ttcatcaatt tctttgtggg gaatacgatc
 601 aattcctcct atcctcctgg ttattcactg cattcgatat cggtgagacg gctgaaggaa
 661 acccaagatg gttttaagtt tttgacagac cagtcctata ttgatgtctt accagaattc
 721 caagattcct accccataaa gtacatacat gccttcgaaa gcaaccattt tatttacttt
 781 ctgactgtcc aaaaggaaac tctagatgct cagacttttc atacaagaat aatcaggttc
 841 tgttccgtag actctgggtt gcactcctac atggaaatgc ccctggaatg catcctgaca
 901 gaaaaaagaa ggaagagatc cacaagggaa gaagtgttta atatcctcca agccgcgtat
 961 gtcagtaaac caggggccaa tcttgctaag caaataggag ctagcccttc tgatgacatt
1021 ctcttcgggg tgtttgcaca aagcaagcca gattctgctg aacctgtgaa tcgatcagca
1081 gtctgtgcat tccccatcaa atatgtcaat gacttcttca acaagattgt caacaaaaac
1141 aacgtgagat gtctccagca tttttacgga cccaaccatg agcactgttt caataggacc
1201 ctgctgagaa actcttccgg ctgtgaagcg cgcagtgacg agtatcggac agagtttacc
1261 acggctttgc agcgcgtcga cttattcatg ggccggctta accaagtgct cctgacatcc
1321 atctccacct tcatcaaagg tgacctcacc attgctaatc tagggacgtc agaaggtcgc
1381 ttcatgcagg tggtgctctc tcgaacagca cacctcactc ctcatgtgaa cttcctcctg
1441 gactcccatc ctgtatctcc agaagttatt gttgagcatc catcaaatca aaatggctat
1501 acattggttg tcacaggaaa gaagatcacc aagattccat tgaatggcct gggctgtgga
1561 catttccaat cctgcagtca gtgcctctct gccccttact ttatacagtg tggctggtgc
1621 cacaatcaat gtgtgcgttt tgatgaatgc cccagcggta catggactca agagatctgt
1681 ctgccggcgg tttataaggt gttccccacc agcgcgcccc ttgaaggagg aacagtgttg
1741 accatatgtg gctgggactt tggattcagg aagaataata aatttgattt aaggaaaacc
1801 aaagttctgc ttggcaacga gagctgtacc ttgaccttaa gcgagagcac gacaaatacg
1861 ttgaaatgca cagttggtcc cgcgatgagt gagcacttca atgtgtctgt aattatctca
1921 aacagtcgag agacgacgca atacagtgca ttctcctatg tagatcctgt aataacaagc
1981 atttctccga ggtacggccc tcaggctgga ggcaccttac tcactcttac tgggaaatac
2041 ctcaacagtg gcaattctag acacatttca attggaggga aaacatgtac tttaaaaagt
2101 gtatcagata gtattcttga atgctacacc ccagcccaaa ctacctctga tgagtttcct
2161 gtgaaattga agattgactt ggctaaccga gagaccagca gcttcagtta ccgggaagac
2221 cccgttgtct atgaaatcca cccgaccaaa tcttttatta gtggtggaag cacaataacg
2281 ggtattggga agaccctgaa ctcggttagc ctcccaaagc tggtaataga tgtgcatgaa
2341 gtgggtgtga actacacagt ggcatgtcag catcgctcaa attcagagat catctgctgc
2401 actactcctt cactgaaaca gctgggcctg caactccccc tgaagaccaa agccttcttc
2461 ctgttagacg ggattctttc caaacacttt gatctcactt atgtgcataa tcctgtgttt
2521 gagccttttg aaaagccagt aatgatctca ataggcaatg aaaatgtagt ggaaattaag
2581 ggaaacaata ttgaccctga agcagttaaa ggtgaagtgt taaaagttgg aaatcagagc
2641 tgcgagagtc tccactggca ctctggagct gtgttgtgta cagtccccag tgacctgctc
2701 aaactgaaca gcgagctaaa tatagagtgg aagcaagcag tctcttcaac tgttcttgga
2761 aaagtgatcg ttcaaccgga tcagaatttt gcaggattga tcattggtgc ggtctcaata
2821 tcagtagtag ttttgttatt atccgggctc ttcctgtgga tgagaaagag aaagcataaa
2881 gatctgggca gtgaattagt tcgctatgac gcaagagtac acactcctca tttggatagg
2941 cttgtaagtg cccgaagtgt aagtccaact acagagatgg tttcaaatga gtctgtagac
3001 tacagagcta cttttccaga agaccagttt cccaactcct ctcagaatgg agcatgcaga
3061 caagtgcaat accctctgac agacctgtcc cctatcctga caagtggaga ctctgatata
3121 tccagcccat tactacaaaa tactgttcac attgacctca gtgctctaaa tccagagctg
3181 gtccaagcag ttcagcacgt agtgattgga cccagcagcc tgattgtgca tttcaatgaa
3241 gtcataggaa gagggcattt tggctgtgtc tatcatggga ctttgctgga caatgacgga
3301 aagaaaattc actgtgctgt gaaatcctta aatagaatca cagatataga agaggtctcc
3361 cagtttctga ctgagggaat catcatgaaa gacttcagcc atcccaatgt tctctcactc
3421 ttgggaatct gcctgaggag tgaagggtct cctctggtgg tcctgcccta tatgaagcat
3481 ggagatctgc gaaatttcat tcgaaacgag actcataatc caactgtgaa agatcttata
3541 ggatttggcc ttcaagtagc caaaggcatg aaatatcttg ccagcaaaaa gtttgtccac
3601 agagacttag ctgcaagaaa ctgcatgttg gatgaaaaat tcactgtcaa ggttgctgat
3661 ttcggtcttg ccagagacat gtacgataaa gagtactata gtgtccacaa caagacgggt
3721 gccaagctac cagtaaagtg gatggcttta gagagtctgc aaacgcagaa gttcaccacc
3781 aagtcagatg tgtggtcctt tggtgtgctc ctctgggagc tcatgacgag aggagcccct
3841 ccttatcccg acgtgaacac atttgatatc actatctacc tgttgcaagg cagaagactc
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3901 ttgcaaccag aatactgtcc agacgccttg tacgaagtga tgctaaaatg ctggcacccc
3961 aaagcggaaa tgcgcccgtc cttttccgaa ctggtctcca ggatatcctc aatcttctcc
4021 acgttcattg gggaacacta cgtccacgtg aacgctactt atgtgaatgt aaaatgtgtt
4081 gctccatatc cttctctgtt gccatcccaa gacaacattg atggcgaggg gaacacatga
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The 1379 amino acid sequence of the murine c-Met polypeptide encoded by the foregoing murine nucleotide sequence is shown below (SEQ ID NO:4).

```
MKAPTVLAPG ILVLLLSLVQ RSHGECKEAL VKSEMNVNMK YQLPNFTAET PIQNVVLHGH
HIYLGATNYI YVLNDKDLQK VSEFKTGPVL EHPDCLPCRD CSSKANSSGG VWKDNINMAL
LVDTYYDDQL ISCGSVNRGT CQRHVLPPDN SADIQSEVHC MFSPEEESGQ CPDCVVSALG
AKVLLSEKDR FINFFVGNTI NSSYPPGYSL HSISVRRLKE TQDGFKFLTD QSYIDVLPEF
QDSYPIKYIH AFESNHFIYF LTVQKETLDA QTFHTRIIRF CSVDSGLHSY MEMPLECILT
EKRRKRSTRE EVFNILQAAY VSKPGANLAK QIGASPSDDI LFGVFAQSKP DSAEPVNRSA
VCAFPIKYVN DFFNKIVNKN NVRCLQHFYG PNHEHCFNRT LLRNSSGCEA RSDEYRTEFT
TALQRVDLFM GRLNQVLLTS ISTFIKGDLT IANLGTSEGR FMQVVLSRTA HLTPHVNFLL
DSHPVSPEVI VEHPSNQNGY TLVVTGKKIT KIPLNGLGCG HFQSCSQCLS APYFIQCGWC
HNQCVRFDEC PSGTWTQEIC LPAVYKVFPT SAPLEGGTVL TICGWDFGFR KNNKFDLRKT
KVLLGNESCT LTLSESTTNT LKCTVGPAMS EHFNVSVIIS NSRETTQYSA FSYVDPVITS
ISPRYGPQAG GTLLTLTGKY LNSGNSRHIS IGGKTCTLKS VSDSILECYT PAQTTSDEFP
VKLKIDLANR ETSSFSYRED PVVYEIHPTK SFISGGSTIT GIGKTLNSVS LPKLVIDVHE
VGVNYTVACQ HRSNSEIICC TTPSLKQLGL QLPLKTKAFF LLDGILSKHF DLTYVHNPVF
EPFEKPVMIS IGNENVVEIK GNNIDPEAVK GEVLKVGNQS CESLHWHSGA VLCTVPSDLL
KLNSELNIEW KQAVSSTVLG KVIVQPDQNF AGLIIGAVSI SVVVLLLSGL FLWMRKRKHK
DLGSELVRYD ARVHTPHLDR LVSARSVSPT TEMVSNESVD YRATFPEDQF PNSSQNGACR
QVQYPLTDLS PILTSGDSDI SSPLLQNTVH IDLSALNPEL VQAVQHVVIG PSSLIVHFNE
VIGRGHFGCV YHGTLLDNDG KKIHCAVKSL NRITDIEEVS QFLTEGIIMK DFSHPNVLSL
LGICLRSEGS PLVVLPYMKH GDLRNFIRNE THNPTVKDLI GFGLQVAKGM KYLASKKFVH
RDLAARNCML DEKFTVKVAD FGLARDMYDK EYYSVHNKTG AKLPVKWMAL ESLQTQKFTT
KSDVWSFGVL LWELMTRGAP PYPDVNTFDI TIYLLQGRRL LQPEYCPDAL YEVMLKCWHP
KAEMRPSFSE LVSRISSIFS TFIGEHYVHV NATYVNVKCV APYPSLLPSQ DNIDGEGNT   1379
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siRNAs

siRNAs suppress gene expression through a highly regulated enzyme-mediated process called RNA interference (RNAi) (Sharp, P.A., *Genes Dev.* 15:485–490 (2001); Bernstein, E *et al.*, *Nature* 409:363–366 (2001); Nykanen, A *et al.*, *Cell* 107:309–321 (2001); Elbashir, S.M. *et al.*, *Genes Dev.* 15:188–200 (2001)). RNAi involves multiple RNA-protein interactions characterized by four major steps: assembly of siRNA with the RNA-induced silencing complex (RISC), activation of the RISC, target recognition and target cleavage. These interactions may bias strand selection during siRNA-RISC assembly and activation, and contribute to the overall efficiency of RNAi (Khvorova, A *et al.*, *Cell* 115:209–216 (2003); Schwarz, DS *et al.* 115:199–208 (2003)))

Two publications that describe preferred approaches and algorithms for selecting siRNA sequences are: Far, RK *et al.*, Nuc Acids Res, 2003, 314417-4424 and Reynolds, A *et al.*, Nature Biotech. 2004, 22:326-330. Far *et al.* suggests options for assessing target accessibility for siRNA and supports the design of active siRNA constructs. This approach can be automated, adapted to high throughput and is open to include additional parameters relevant to the biological activity of siRNA. To identify siRNA-specific features likely to Contribute to efficient processing at each of the steps pf RNAi

noted above, Reynolds *et al.*, *supra* performed a systematic analysis of 180 siRNAs targeting the mRNA of two genes. Eight characteristics associated with siRNA functionality were identified: low G/C content, a bias towards low internal stability at the sense strand 3'-terminus, lack of inverted repeats, and sense strand base preferences (positions 3, 10, 13 and 19). Application of an algorithm incorporating all eight criteria significantly improves potent siRNA selection. This highlights the utility of rational design for selecting potent siRNAs that facilitate functional gene knockdown.

Candidate siRNA sequences against mouse and human *c-met* are selected using a process that involves running a BLAST search against the sequence of *c-met*, and selecting sequences that "survive" to ensure that these sequences will not be cross matched with any other genes.

siRNA sequences selected according to such a process and algorithm may be cloned into an expression plasmid and tested for their activity in abrogating Met function in Met-expressing cells of the appropriate animal species. Those sequences that show RNAi activity are preferably recloned into a replication-defective human adenovirus serotype 5 (Ad5).

One reason for selection of this viral vector the high titer obtainable (in the range of 10^{10}) and therefore the high multiplicities-of infection that can be attained. For example, infection with 100 infectious units/ cell ensures all cells are infected. Another advantage of this virus is the high susceptibility and infectivity and the host range (with respect to cell types). Even if expression is transient, cells can go through multiple replication cycles before Met activity recovers (see Examples). Moreover, some tumors undergo apoptosis in response to expression of the present siRNAs, so that even transient expression is adequate to kill the cells.

Preferred constructs described in the Examples are the following:

(a) si-mMet-Ad5^{178} which exerted the most dramatic effect on DA3 mouse mammary adenocarcinoma cells and M114 fibroblast cells,

(b) si-hMet-Ad5^{221} which had the strongest effects on human glioblastoma cells (using the line DBTRG as an example), human prostate cancer cells (using PC-3 as an example) and human gastric cancer cells (using MKN45 as an example).

Preferred viral vectors are those with prolonged suppressive effect against Met, lasting beyond passage of the cells in culture.

In a most preferred embodiment, the inhibitory molecule is a double stranded nucleic acid (preferably an RNA), used in a method of RNA interference. RNA interference is the sequence-specific degradation of homologues in an mRNA of a targeting sequence in an siNA. As used herein, the term siNA (small, or short, interfering nucleic acid) is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi (RNA interference), for example short (or small) interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA

(miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically-modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), translational silencing, and others. Long double stranded interfering RNAs, such a miRNAs, appear to tolerate mismatches more readily than do short double stranded RNAs. In addition, as used herein, the term RNAi is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post transcriptional gene silencing, or an epigenetic phenomenon. For example, siNA molecules of the invention can be used to epigenetically silence genes at both the post-transcriptional level or the pre-transcriptional level. In a non-limiting example, epigenetic regulation of gene expression by siNA molecules of the invention can result from siNA mediated modification of chromatin structure and thereby alter gene expression (see, for example, Allshire (2002) *Science 297*, 1818-1819; Volpe *et al.* (2002) *Science 297*, 1833-1837; Jenuwein (2002) *Science 297*, 2215-2218; and Hall *et al.* (2002) *Science 297*, 2232-2237.)

An siNA can be designed to target any region of the coding or non-coding sequence of an mRNA. An siNA is a double-stranded polynucleotide molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region has a nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary. The siNA can be assembled from a single oligonucleotide, where the self-complementary sense and antisense regions of the siNA are linked by means of a nucleic acid based or non-nucleic acid-based linker(s). The siNA can be a polynucleotide with a hairpin secondary structure, having self-complementary sense and antisense regions. The siNA can be a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the circular polynucleotide can be processed either *in vivo* or *in vitro* to generate an active siNA molecule capable of mediating RNAi. The siNA can also comprise a single stranded polynucleotide having nucleotide sequence complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof (or can be an siNA molecule that does not require the presence within the siNA molecule of nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof), wherein the single stranded polynucleotide can further comprise a terminal phosphate group, such as a 5'-phosphate (see for example Martinez *et al.* (2002) *Cell 110*, 563-574 and Schwarz *et al.* (2002) *Molecular Cell 10*, 537-568), or 5',3'-diphosphate.

In certain embodiments, the siNA molecule of the invention comprises separate sense and antisense sequences or regions, wherein the sense and antisense regions are covalently linked by

nucleotide or non-nucleotide linkers molecules as is known in the art, or are alternately non-covalently linked by ionic interactions, hydrogen bonding, Van der Waal's interactions, hydrophobic interactions, and/or stacking interactions. Some preferred siRNAs are discussed in the Examples.

As used herein, siNA molecules need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides. In certain embodiments, the short interfering nucleic acid molecules of the invention lack 2'-hydroxy (2'-OH) containing nucleotides. In certain embodiments, short interfering nucleic acids do not require the presence of nucleotides having a 2'-hydroxy group for mediating RNAi and as such, short interfering nucleic acid molecules of the invention optionally do not include any ribonucleotides (*e.g.*, nucleotides having a 2'-OH group). Such siNA molecules that do not require the presence of ribonucleotides within the siNA molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally, siNA molecules can comprise ribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide positions. The modified short interfering nucleic acid molecules of the invention can also be referred to as short interfering modified oligonucleotides "siMON." Other chemical modifications, *e.g.*, as described in PCT/US03/05346 and PCT/US03/05028, can be applied to any siNA sequence of the invention.

Preferably a molecule mediating RNAi has a 2 nucleotide 3' overhang. If the RNAi molecule is expressed in a cell from a construct, for example from a hairpin molecule or from an inverted repeat of the desired sequence, then the endogenous cellular machinery will create the overhangs.

Considerations to be taken into account when designing an RNAi molecule include, *e.g.*, the sequence to be targeted, secondary structure of the RNA target and binding of RNA binding proteins. Methods of optimizing siRNA sequences will be evident to the skilled worker. Typical algorithms and methods are described in Vickers *et al.* (2003) *J Biol Chem 278*:7108-7118; Yang *et al.* (2003) *Proc Natl Acad Sci USA 99*:9942-9947; Far *et al.* (2003) *Nuc. Acids Res. 31*:4417-4424; and Reynolds *et al.* (2004) *Nature Biotechnology 22*:326-330.

Methods of making siRNAs are conventional. *In vitro* methods include processing the polyribonucleotide sequence in a cell-free system (*e.g.*, digesting long dsRNAs with RNAse III or Dicer), transcribing recombinant double stranded DNA *in vitro*, and, preferably, chemical synthesis of nucleotide sequences homologous to cMet sequence. See, *e.g.*, Tuschl *et al.* (1999) *Genes & Dev. 13*:3191-3197.

In vivo methods include

(1) transfecting DNA vectors into a cell such that a substrate is converted into siRNA *in vivo*. See, for example, Kawasaki *et al.* (2003) *Nucleic Acids Res 31*:700-707; Miyagishi *et al.* (2003) *Nature Biotechnol 20*:497-500; Lee *et al.* (2002) *Nature Biotechnol 20*:500-505:Brummelkamp *et al.*

(2002) *Science 296*:550-553; McManus *et al.* (2002) *RNA 8*:842-850; Paddison *et al.* (2002) *Gene Dev 16*:948-958; Paddison *et al.* (2002) *Proc Natl Acad Sci USA 99*:1443-1448); Paul *et al.* (2002) *Nature Biotechnol 20*:505-508; Sui *et al.* (2002) *Proc Natl Acad Sci USA 99*:5515-5520; Yu *et al.* (2002) *Proc Natl Acad Sci USA 99*:6047-6052];

(2) expressing short hairpin RNAs from plasmid systems using RNA polymerase III (pol III) promoters. See, for example, Kawasaki *et al.*, *supra;* Miyagishi *et al.*, *supra*; Lee *et al.*, *supra*; Brummelkamp *et al.*, *supra*; McManus *et al.*, *supra*), Paddison *et al.*, *supra* (both); Paul *et al.*, *supra*, Sui *et al.*, *supra*; and Yu *et al.*, *supra*; and/or

(3) expressing short RNA from tandem promoters. See, for example, Miyagishi *et al.*, *supra;* Lee *et al.*, *supra*).

When synthesized *in vitro*, a typical μM scale RNA synthesis provides about 1 mg of siRNA, which is sufficient for about 1000 transfection experiments using a 24-well tissue culture plate format. In general, to inhibit cMet expression in cells in culture, one or more siRNAs can be added to cells in culture media, typically at about 1 ng/ml to about 10 μg siRNA/ml.

For reviews and more general description of inhibitory RNAs, see Lau *et al.* (2003 Aug) *Sci Amer* pp 34-41; McManus *et al.* (2002) *Nature Rev Genetics 3*, 737-747; and Dykxhoorn *et al.* (2003) *Nature Rev Mol Cell Bio 4:* 457-467. For further guidance regarding methods of designing and preparing siRNAs, testing them for efficacy, and using them in methods of RNA interference (both *in vitro* and *in vivo*), see, *e.g.*, Allshire (2002) *Science 297*:1818-1819; Volpe *et al.* (2002) *Science 297*:1833-1837; Jenuwein (2002) *Science 297*:2215-2218; Hall *et al.* (2002) *Science 297* 2232-2237; Hutvagner *et al.* (2002) *Science 297*:2056-60; McManus *et al.* (2002) *RNA 8*:842-850; Reinhart *et al.* (2002) *Genes Dev. 16*:1616-1626; Reinhart *et al.* (2002) *Science 297*:1831; Fire *et al.* (1998) *Nature 391*:806-811:Moss (2001) *Curr Biol 11*:R772-5:Brummelkamp *et al.* (2002) *Science 296*:550-553; Bass (2001) *Nature 411* 428-429; and Elbashir *et al.* (2001) *Nature 411*:494-498; US Pat. 6,506,559; Published US Pat App. 20030206887; and PCT applications WO99/07409, WO99/32619, WO 00/01846, WO 00/44914, WO00/44895, WO01/29058, WO01/36646, WO01/75164, WO01/92513, WO 01/29058, WO01/89304, WO01/90401, WO02/16620, and WO02/29858.

Ribozymes and siNAs can take any of the forms, including modified versions, described for antisense nucleic acid molecules; and they can be introduced into cells as oligonucleotides (single or double stranded), or in an expression vector.

In a preferred embodiment, an antisense nucleic acid, siNA (*e.g.*, siRNA) or ribozyme comprises a single stranded polynucleotide comprising a sequence that is at least about 90% (*e.g.*, at least about 93%, 95%, 97%, 98% or 99%) identical to a segment of SEQ ID NO: 1, or 3, or a complement thereof.

As used herein, a DNA and an RNA encoded by it are said to contain the same "sequence," taking into account that the thymine bases in DNA are replaced by uracil bases in RNA.

Active variants (*e.g.*, length variants, including fragments; and sequence variants) of the nucleic acid-based inhibitors discussed herein are included. An "active" variant is one that retains an activity of the inhibitor from which it is derived (preferably the ability to inhibit expression). It is routine to test a variant to determine for its activity using conventional procedures.

As for length variants, an antisense nucleic acid or siRNA may be of any length that is effective for inhibition of a gene of interest. Typically, an antisense nucleic acid is between about 6 and about 50 nucleotides (*e.g.*, at least about 12, 15, 20, 25, 30, 35, 40, 45 or 50 nt), and may be as long as about 100 to about 200 nucleotides or more. Antisense nucleic acids having about the same length as the gene or coding sequence to be inhibited may be used. When referring to length, the terms bases and base pairs (bp) are used interchangeably, and will be understood to correspond to single stranded (ss) and double stranded (ds) nucleic acids. The length of an effective siNA is generally between about 15 bp and about 29 bp in length, preferably between about 19 and about 29 bp (*e.g.*, about 15, 17, 19, 21, 23, 25, 27 or 29 bp), with shorter and longer sequences being acceptable. Generally, siNAs are shorter than about 30 bases to prevent eliciting interferon effects. For example, an active variant of an siRNA having, for one of its strands, the 19 nucleotide sequence of any of SEQ ID NO: 9-15 herein can lack base pairs from either, or both, of ends of the dsRNA; or can comprise additional base pairs at either, or both, ends of the ds RNA, provided that the total of length of the siRNA is between about 19 and about 29 bp, inclusive. One embodiment of the invention is an siRNA that "consists essentially of" sequences represented by SEQ ID NO: 9-15 or complements of these sequence. The term "consists essentially of" is an intermediate transitional phrase, and in this case excludes, for example, sequences that are long enough to induce a significant interferon response. An siRNA of the invention may consist essentially of between about 19 and about 29 bp in length.

As for sequence variants, it is generally preferred that an inhibitory nucleic acid, whether an antisense molecule, a ribozyme (the recognition sequences), or an siNA, comprise a strand that is complementary (100% identical in sequence) to a sequence of a gene that it is designed to inhibit. However, 100% sequence identity is not required to practice the present invention. Thus, the invention has the advantage of being able to tolerate naturally occurring sequence variations, for example, in human c-met, that might be expected due to genetic mutation, polymorphism, or evolutionary divergence. Alternatively, the variant sequences may be artificially generated. Nucleic acid sequences with small insertions, deletions, or single point mutations relative to the target sequence can be effective inhibitors.

The degree of sequence identity may be optimized by sequence comparison and alignment algorithms well-known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). At least about 90% sequence identity is preferred (*e.g.*, at least about 92%, 95%, 98% or 99%), or even 100% sequence identity, between the inhibitory nucleic acid and the targeted sequence of targeted gene.

Alternatively, an active variant of an inhibitory nucleic acid of the invention is one that hybridizes to the sequence it is intended to inhibit under conditions of high stringency. For example, the duplex region of an siRNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript under high stringency conditions (*e.g.*, 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C, hybridization for 12-16 hours), followed generally by washing.

Murine DA3 cells, when not infected with the viral vector of the invention, respond to HGF stimulation by scattering. In contrast, the same cells infected with a viral vector comprising the present siRNA sequences do not respond, or respond more weakly, to HGF.

Met+ tumor cells infected with the siRNA vectors of the present invention have significantly reduced proliferative and invasive activity, and undergo enhanced apoptotic cell death. Infection with a viral vector comprising the siRNA of the present invention results in inhibition of phosphorylation of Met and its downstream pathways, manifest as reduced phosphorylation of Akt and p44/42 MAPK.

Delivery and expression of the siRNA compositions of the present invention inhibit (a) *in vivo* tumorigenesis *de novo*, and (b) growth of existing Met+ tumor/cancer cells. These capabilities have been exemplified by showing that DA3 cells infected with si-mMet-Ad5^{178} adenovirus *in vitro* and inoculated into mice sc are inhibited in their ability to form tumors. Moreover, treatment of subjects with si-mMet-Ad5^{178} adenovirus resulted in a dramatic reduction in the tumor size. Thus the constructs of the present invention are useful for "nucleic acid" or "gene" therapy of Met-expressing cancer *in vivo*.

Therapeutic Compositions and Methods

The preferred animal subject of the present invention is a mammal. The invention is particularly useful in the treatment of human subjects. By the term "treating" is intended the administering to a subject of an effective dose of a pharmaceutical composition comprising an c-met siRNA or other c-Met specific siNA, preferably in the form of a viral vector that comprises (a) an expression construct of the siRNA operatively linked to a promoter, and (b) a pharmaceutically acceptable excipient or carrier. Preferred doses are between about 1 ng and 100 mg/kg body weight and may be administered once or repeatedly. The composition such as the viral vector, may be administered by any acceptable route, *e.g.*

injected or infused systemically (preferably intravenously or intramuscularly), injected or instilled regionally, (*e.g.*, subcutaneously, intrabronchially) or locally (*e.g.*, intradermally, intrathecally). One preferred route is direct intratumoral administration.

The invention further relates to use of the c-met siRNA, other c-Met specific siNA, c-Met specific siNA expression constructs and viral vectors comprising such expression constructs for the manufacture of medicaments for use in therapeutic methods as herein described.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE 1

Materials and Methods

Cell lines. DA3 cells (poorly differentiated mouse mammary adenocarcinoma) (Firon *et al.*, *supra*), M114 cells (NIH3T3 cells stably-transfected with mouse met and mouse HGF/SF), SK-LMS-1 human leiomyosarcoma cells (Zhang *et al.*, *supra*), PC-3 human prostate cancer cells (Humphrey, PA *et al., Am J Pathol 147:*386-396, 1995), DBTRG human glioblastoma cells (Koochekpour, S *et al., Canc Res 57:*5391-5398, 1997), Madin-Darby canine kidney epithelial cells (MDCK) and TR6LM canine prostate carcinoma cells of a line established from a spontaneous lung metastasis, were grown in DMEM (Gibco™, Invitrogen Cooperation) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone). Ovarian cancer cell lines CAOV3, ES2, and OVMZ6 (Munich) were similarly grown and the SKOV-3 line was maintained in suspension culture. MKN45 human gastric cancer cells (Kitamura, S *et al., Biochem Biophys Res Commun 265:*453-456, 1999) were grown in RPMI-1640 (Gibco™, Invitrogen Cooperation) supplemented with 10% FBS.

Met siRNA expression plasmids. The mU6 pro vector containing the mouse U6 promoter (Yu, JY *et al., Proc Natl Acad Sci USA 99:*6047-6052, 2002) or the pSilencer 1.0-U6 siRNA expression vector (Ambion, Inc.) was used for the construction of mouse and human met siRNA expression plasmids. The siRNA target finder and design tool provided by Ambion, Inc. was used for selecting the siRNA sequences (see footnote). Four mouse and three human candidate siRNA sequences were selected from met mRNA sequences (Table 2). These sequences survived a BLAST search to ensure that there were no cross matches to other genes. The oligonucleotides that encode the Met mRNA 19-mer hairpin sequences were cloned into an expression vector plasmid (the BbsI and XbaI sites in the mU6 pro vector, and the ApaI and EcoRI sites in the pSilencer 1.0-U6 vector) and tested for Met suppression activity in either mouse or human cells, respectively.

Construction of c-met siRNA adenoviruses. The AdEasy™ Adenoviral Vector System (human adenovirus serotype 5, or Ad5, by Stratagene) was used. First, the selected siRNA sequences were recloned with the U6 promoter into a pShuttle vector. As a mock vector, U6 promoter without the siRNA sequence was used. Then, pShuttle vectors containing siRNA sequences were linearized with PmeI and cotransformed with pAdEasy-1 into BJ5183 cells by electroporation. Positive (homologously recombined) clones were selected and confirmed by PacI digestion. Plasmids with the correct insert were transformed into TOP10 competent cells and amplified; plasmid DNA was extracted using a QIAGEN Plasmid Midi Kit. The linearized adenoviral DNA was prepared by digesting the plasmid with PacI, after which it was transfected into the packaging cell line HEK293. Transfected cells were cultured for 7 d and the virus was harvested. After repeating one more amplification cycle, a large-scale amplification was performed by using a large-scale-production tissue culture plate (Cell Factory, Nunclon). Purification of the virus was performed according to Herz *et al.*, *Proc Natl Acad Sci U S A 90:*2812-2816, 1993. The virus titer was evaluated by plaque assay or end-point dilution.

Adenovirus infection. Cells at 75–80% confluence were exposed to c-met siRNA adenovirus diluted in a small volume of growth medium (+ 10% FBS) at a multiplicity of infection (moi) of 10 to 100 for 4 h at 37°C. After 4 h, fresh complete growth medium was added and the cells were cultured in a CO_2 incubator at 37°C. After 2 to 4 days in culture, the infected cells were collected for Western blotting, proliferation assays, invasion assays, or morphological analyses.

Western blot analysis. Cell extracts were separated by SDS-PAGE and transferred to PVDF membranes (Invitrogen). The membranes were incubated with antibodies against Met (SP260: sc-162, Santa Cruz; C-28: sc-161, Santa Cruz); phospho-Met ($Tyr^{1234/1235}$ rabbit polyclonal antibodies, Cell Signaling); p44/42 MAPK (rabbit polyclonal antibodies, Cell Signaling); phospho-p44/42 MAPK (Thr^{202}/Tyr^{204} rabbit polyclonal antibodies, Cell Signaling); phospho-Akt (Ser^{473}, 587F11, Cell Signaling); or β-actin (AC-15: ab6276, abcam), followed by HRP-conjugated secondary antibodies (Santa Cruz). After incubation with ECL reagent (Amersham Biosciences), chemiluminescence signals were photographed and quantitated by image analysis.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated using TRIzol reagent (Invitrogen). Reverse transcription was performed using 1 μg RNA and the SuperScript™ II RNase H^- Reverse Transcriptase (Invitrogen). One microliter of the RT product was used for amplification of c-met or β-actin genes. The primers used were

met-sense, 5'-AGCCAGTAATGATCTCAATAG-3' (SEQ ID NO:5);

met-antisense, 5'-TCAGGATAGGGGACAGGT-3' (SEQ ID NO:6) ;

β-actin sense, 5'-CGTGACATCAAAGAGAAGCTGTG-3' (SEQ ID NO:7); and

β-actin antisense, 5'-GCTCAGGAGGAGCAATGATCTTGA-3' ((SEQ ID NO:8).

The PCR conditions were 95°C for 5 min, followed by 35 cycles of 95°C, 1 min; 55°C, 1 min; and 72°C, 1 min. The final extension was 72°C for 5 min. Values were quantified using Scion image software and normalized to β-actin.

Scatter assay. Cells were seeded into six-well culture plates and treated with low-serum DMEM for 24 h (Stoker, M *et al., Nature 327:*239-242, 1987). Then assay medium containing HGF/SF (100 ng/ml) was added to the cells and they were incubated overnight. Cell scattering was observed microscopically.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay. In Situ Cell Death Detection Kit, POD (Roche Diagnostics GmbH, Germany) was used for the TUNEL technology. Cells (1000/well) were seeded into 96-well microplates. After adhering, cells were infected with mU6-Ad5 mock or Met siRNA viruses at moi of 10, 50, and 100. Three and six days after infection, cells were fixed with 4% buffered formalin and processed for TUNEL assay according to manufacturer's instructions. Briefly, intrinsic peroxidase was blocked with 3% H_2O_2 in methanol for 10 min, and cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. TdT reaction was performed for 60 min at 37°C in a humidified atmosphere. Peroxidase substrate kit DAB (Vector Laboratories, Inc., CA) was used for the color development. Morphological evaluations were by light microscopy.

Sub-G_1 fraction analysis. Cells ($5x10^4$/well) were seeded into 6-well plates and infected with Met siRNA viruses at different moi (10, 50, and 100). Three and six days after infection, cells were harvested and processed for flow cytometric analysis. The suspensions of tumor cells were prepared using the detergent–trypsin method (Vindelov *et al., Cytometry 3:*323-327, 1983) and stained with propidium iodide. Measurement of DNA cellular contents was performed with a flow cytometer (Becton–Dickinson). Fractions with DNA content below the 2C peak (sub-G_1 fraction) were calculated using the CELLQuest software package, and taken as apoptotic fractions.

In vitro invasion. Invasion assays were performed using a 24-well Matrigel™ invasion chamber plates (Becton-Dickinson) (Jeffers, M *et al., Mol Cell Biol 16:*1115-1125, 1996). Cells infected with Met siRNA adenovirus for three days were tested. Lower and upper wells were separated by 8μm pore filters coated with Matrigel. In the lower wells was placed 0.75 ml 0.1% BSA-DMEM with or without HGF (100 ng/ml). Cells ($2.5x10^4$) suspended in 0.5 ml 0.1% BSA-DMEM were placed in the upper wells and plates were incubated for 24 h in a CO_2 incubator. Non-invading cells in the upper wells were removed with cotton swabs and invading cells that migrated to the lower surface of the filter were fixed with methanol and stained with Diff-Quik® stain. The number of infiltrating cells was counted under a microscope.

Cell proliferation assay. Cells (1000/well) were seeded into 96-well microplates. After adhering, cells were infected with mU6-Ad5 mock or Met siRNA viruses at moi of 10, 50, and 100 and incubated for 2 days. Thereafter, cells were washed and the medium replaced with 0.1% BSA DMEM without FBS and incubated one more day. The cells were stimulated with HGF/SF (100 ng/ml) and incubated for 24 h. after which 10 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) were added to the wells and the plates incubated for 4 h. Subsequently, medium was removed and the produced dye was dissolved in 100 μl of DMSO. The formazan reaction product was determined with an ELISA plate reader (at a wavelength of 540 nm.

In vivo tumor formation. DA3 cells were infected with met siRNA adenovirus at moi of 100. Three days later, the cells were trypsinized, washed twice, and resuspended in Hanks' balanced salt solution (HBSS) supplemented with 0.6% lactalbumin hydrolysate. Cells, 10^5 in 0.1 ml HBSS, were injected sc into the right flank of BALB/c mice. Tumor formation was monitored twice weekly.

Activity of c-met siRNA adenovirus in vivo. DA3 cells (10^5) were inoculated sc into the right flank of BALB/c mice. After 3 and 7 days, c-met siRNA adenovirus ($4x10^7$ infectious unit in 0.1 ml) was injected directly into the tumor. Tumor size was followed for 24 days. The mean and SD were calculated for each group, and statistical significance was evaluated using Student's t-test.

EXAMPLE 2

c-met siRNA Adenoviruses and Met Expression

Four c-met siRNA adenoviruses were produced for mouse cells and three for human cells and three for canine cells (Table 2). In addition, mock viruses were prepared containing the mU6 promoter but no siRNA sequence (mU6-Ad5). M114 mouse NIH3T3 cells transformed with mouse *met* and HGF/SF (Rong, S *et al., Mol Cell Biol 12:*5152-5158, 1992) were used. The cells were infected with four different mouse c-*met* siRNA adenovirus preparations (si-mMet-Ad5 $^{57, 60, 110, \text{and } 178}$; see Table 2) at moi=10, 50, and 100. Met expression was determined on 3 days after infection by Western blot (Fig. 1A). The expression of Met protein was dramatically suppressed by si-mMet-Ad5^{57} and si-mMet-Ad5^{178} at moi=50 and 100; mU6-Ad5 infected cells showed no effect on Met expression. Since M114 cells produce a high level of Met, both the p140 (mature) and p170 (pro-form) Met were observed. The si-mMet-Ad5 adenoviruses reduced the quantity of both p140 and p170 Met proteins.

Table 2. Design of c-*met* siRNA adenovirus constructs

Species	Adenovirus construct	siRNA gene position in c-*met* mRNA	19-mer Target sequence	SEQ ID NO:
Mouse	si-mMet-Ad5^{57}	950	GCCGCGTATGTCAGTAAAC	9
	si-mMet-Ad5^{60}	988	GCAAATAGGAGCTAGCCCT	10
	si-mMet-Ad5^{110}	1839	GCGAGAGCACGACAAATAC	11
	si-mMet-Ad5^{178}	2762	GTGATCGTTCAACCGGATC	12
Human	si-hMet-Ad5^{16}	415	GACCTTCAGAAGGTTGCTG	13
	si-hMet-Ad5^{62}	1236	GCCAGATTCTGCCGAACCA	14
	si-hMet-Ad5^{221}	3310	GTGCAGTATCCTCTGACAG	15
Dog	si-dMet-Ad5^{111}	1904	GTGAGAGCACAACAAATAT	16
	si-dMet-Ad5^{197}	2827	GTAATAGTTCAACCAGATC	17
	si-dMet-Ad5^{222}	3130	GTACAATATCCTCTGACGG	18
control	mU6-Ad5 (mock)	—	—	

The effect of human c-*met* siRNA adenoviruses (si-hMet-Ad5 $^{16, 62, \text{and } 221}$) were tested on DBTRG human glioblastoma cells, PC-3 prostate cancer cells, and MKN45 human gastric cancer cells. All these cell lines express high levels of Met protein. Met expression was markedly suppressed by all three si-hMet-Ad5 forms at moi=10 to 100 (Fig. 1B and 1C). The inhibitory effect was strongest with si-hMet-Ad5^{221}. Met is constitutively activated in PC-3 cells and all three forms of si-hMet Ad5 significantly suppressed Met expression. When PC-3 cells were exposed to a mixture of all three forms (si-hMet-Ad5$^{16, 62, 221}$) at 33.3 moi each, the RNAi effect was similar to the effect observed with si-hMet-Ad5^{221} alone at 100 moi (Fig. 1C left). MKN45 cells express extremely high levels of Met, and, again, Met expression was dramatically suppressed by si-hMet-Ad5^{221} at moi=50 or 100 (Fig. 1D). The effect of infection with si-hMet-Ad5^{221} was tested on four human ovarian carcinoma cell lines: CAOV3, ES-2, SKOV-3 and OVMZ6 (Munich). Results shown in Figures 11A-11D indicate that Met expression was markedly reduced in ES-2 and SKOV-3, and even more dramtically suppressed in OVMZ6. Thus these inhibitory effects of Met-specific RNAi constructs are manifest on a variety of Met-expressing cancers of distinct tissue origin.

EXAMPLE 3

Effects of *met* RNAi on Cell Morphology

M114 cells are autocrine for HGF/SF and Met (Rong, S *et al., Mol Cell Biol 12:*5152-5158, 1992). Tests were done to determine whether suppression of Met expression and disruption of the autocrine loop would morphologically change the cells to a non-transformed appearance. After infection

with si-mMet-Ad5 viruses, the M114 cells became less refractile and adhered tightly to the culture dishes (Fig. 2A, top), suggesting reversion to their NIH-3T3 (untransformed) phenotype. Non-infected control or mock virus–infected M114 cells retained spindle shapes and remained refractile. The RNAi effect on the M114 cell morphology is moi-dependent, and cell growth was significantly suppressed in si-mMet-Ad5^{178}-infected cells at moi=50 or 100. The cells were more adherent and less refractile at higher moi (Fig. 2A, middle). Since cell density differed between the cells at different moi's, the cells were trypsinized and reseeded at a fixed concentration, and morphology was checked 24 h later. Again the M114 cells at moi=100 showed the most refractile and adherent appearance (Fig. 2A bottom).

Morphological change in DBTRG cells was more dramatic (Fig. 2B). Uninfected control cells revealed spindle shaped cell bodies and formed a multicellular palisade pattern. In contrast, si-hMet-Ad5^{221}-infected cells showed a wider and rounder cytoplasm. Many cells lost their adherence and rounded up from the plate bottom at moi=50 to 100.

EXAMPLE 4

Specificity of Met Expression in DA3 Cells after Infection with si-mMet-Ad5

To test the specificity of the si-Met-Ad5 adenoviruses, DA3 mouse mammary adenocarcinoma cells were infected with siRNA adenoviruses specifically designed either for mouse Met (si-mMet-Ad5^{178}) or for human Met (si-hMet-Ad5^{221}), and the level of Met expression was observed at day 3 (Fig. 3A). Met expression was strongly suppressed only in the cells infected with si-mMet-Ad5^{178} (although the si-hMet-Ad5^{221} virus worked well in human cancer cell lines. See Fig. 1B, C, D.

The effect of si-mMet–Ad5 adenovirus was also confirmed at the mRNA level by RT-PCR (Fig. 3B). A dramatic reduction in *met* mRNA expression was observed in DA3 cells infected with si-mMet-Ad5^{178} virus. In contrast, no reduction in *met* mRNA was observed in mock–infected cells (mU6-Ad5).

EXAMPLE 5

Effects of *met* RNAi on Cell Scattering

DA3 cells were tested for scattering activity in response to HGF/SF (Firon *et al.*, *supra*) after infection with si-mMet-Ad5. Non-infected (control) DA3 cells showed dramatic scattering after HGF/SF stimulation (Fig. 4A) as did mock–infected cells. However, cell scattering was suppressed in all cells that were infected with si-mMet-Ad5 viruses 57, 110 and 178 (Fig. 4B).

EXAMPLE 6

RNAi Persistence after Cell Passage

The inventors determined how long the RNAi effects continued after cell passage. M114 cells were infected with si-mMet-Ad5^{178} at moi=100 and Met expression was determined at 3-d intervals for

up to nine days (Fig. 5A). Three days after infection, p140 and p170 Met were dramatically suppressed (<5% expression) compared with non-infected or mock–infected cells. Cells were trypsinized and reseeded at lower concentration, and after culture for 3 d ,Met expression remained strongly suppressed (passage 1, Fig. 5A). After another passage, Met expression was still markedly reduced but increased slightly vs. passage 1. These results suggest that not only is the Met expression suppressed in the primary infected cells, but the RNAi effect persists for several cell cycles.

SK-LMS-1 cells were similarly tested and found to be refractory to si-hMet-Ad5 infection compared to other human cell lines. Met expression was not suppressed even at moi=100 at day 3. However, after cell passage Met expression was efficiently suppressed (Fig. 5B). Collectively, these results show that the RNAi lasts through multiple cell divisions.

DA3 cells were infected with si-mMet-Ad5 viruses at moi=100 and Met expression was determined for 3 d (Fig. 5C). At 24 h, the Met expression decreased in all infected cell groups but not in control or mock virus (mU6-Ad5)-infected cells. At 48 and 72 h, Met reduction was more dramatic and in all cases was 25% or less relative to control cells. si-mMet-Ad5^{178} was most effective, and by 72 h Met expression was nil. A mixture of the three, si-mMet-Ad5$^{57, 110, 178}$, was no more effective than si-mMet-Ad5^{178} alone. Similarly, Met expression was tested in the DA3 cells after cell passage. RNAi lasted beyond one passage. However, the RNAi effect was so dramatic that the cells could not survive under the influence of the potent RNAi effect.

EXAMPLE 7

Met RNAi Induces Apoptotic Cell Death

Because DA3 cells could not maintain viability after a second passage following infection with si-mMet-Ad5^{178}, the possibility that they underwent apoptotic cell death was tested. DA3 cells were infected with si-mMet-Ad5^{178} at different moi (10, 50, and 100), and apoptotic changes were assessed by TUNEL assay (Fig. 6). Three days after infection, cell growth was suppressed dose-dependently, and darkly stained apoptotic cells were observed in the si-mMet-Ad5^{178}-infected group. At day 6, the ratio of TUNEL-positive cells increased dramatically, and many apoptotic cells (>30 cells/high power field) were observed at moi=100. A similar but less pronounced apoptotic change was observed in MKN45 and PC-3 cells after infection with si-hMet-Ad5^{221} (Table 3). Although the frequency was lower than these three cancer cell lines, DBTRG, SK-LMS-1, and SK-HGF cells also showed an apoptotic change. However, M114 cells (NIH-3T3 cells transformed with mouse *met* and HGF/SF), did not show an obvious increase in the TUNEL-positive cells, suggesting that Met expression is not essential in the survival of this cell line. Growth inhibition of Met siRNA adenovirus-infected cells seemed to correlate with the extent of apoptotic changes (Table 3). Interestingly, proliferation of SK-LMS-1-HGF cells was

more strongly suppressed than that of SK-LMS-1 cells, suggesting that infection with si-Met-Ad5 virus effectively blocked the autocrine loop of HGF/SF-Met stimulation.

To quantitate apoptosis in Met siRNA adenovirus-infected cells, sub-G_1 fraction analysis was performed (Fig. 7). Apoptosis was induced in an infectious dose-dependent manner. Among the cell lines tested, DA3 cells were most susceptible to apoptosis and the sub-G_1 fraction 6 days after infection reached 67.3±3.2% at moi=100. There was no reduction in the viability of the DA3 cells infected with mock mU6-Ad5 virus at moi=100 at day 6. MKN45 cells were also sensitive to apoptosis and sub-G_1 ratio at moi=100 reached 43.1±2.7% at day 6. DBTRG cells were less susceptible to apoptosis than DA3, MKN45, and PC-3, but sub-G_1 fraction at moi=100 showed yet 10.4±0.5 % and was significant when compared to non-infected control cells.

Table 3. Susceptibility to apoptosis, growth inhibition, and morphological changes after infection with Met siRNA adenovirus

Cells	Apoptosis*	Growth inhibition** (cell confluence)	Morphological changes***
DA3	+++	+++	-
M114	+/-	+	+ (less refractile, flatter)
PC-3	++	++	-
DBTRG	+	++	+ (less spindly, rounder)
MKN45	++	+++	-
SK-LMS-1	+	+	-
SK-HGF#	+	++	-

* Frequency of apoptosis was evaluated by TUNEL assay. Cells were infected with Met siRNA adenovirus at moi=100 and cultured for 6 days. +/-: <5 apoptotic cells/high power field; +: 5 –10 apoptotic cells; ++: 11-30 apoptotic cells; +++: >30 apoptotic cells.

** 10^4 cells were plated in 96-well plates and infected with Met siRNA adenovirus at moi=100. Growth inhibition was estimated at day 3 after infection. Cell morphology was observed using phase contrast microscopy. All control cells reached confluence before day 3. +: confluent but cell density differs from control; ++: 60-70% confluent; +++: less than 50% confluent.

*** Morphological changes were evaluated using phase contrast microscopy (see also Fig. 2).

SK-HGF cells are SK-LMS-1 cells that have been made autocrine for HGF/SF (Jeffers, 1996, *supra*

EXAMPLE 8

met RNAi on TR6M Canine Prostate Carcinoma Cells and Nontransformed MDCK Cells

The effects of three different si-dMET-Ad5 viruses were tested at moi=50 and 100 on nontransformed canine MDCK cells and TR6LM prostate carcinoma cells. All three viruses were very effective at knocking down Met expression in both MDCK and TR6LM cells (Fig. 10A). The order of Met suppression in MDCK was si-dMet-Ad5^{111}> si-dMet-Ad5^{222} > si-dMet-Ad5^{197}. In TR6LM cells, Met expression was almost completely inhibited by each of the three siRNA constructs. The growth and viability of the TR6LM canine prostate cancer cells was adversely affected whereas the viability and

proliferation of MDCK cells was not obviously affected (not shown). However, MDCK cell scattering was suppressed and the scattering response paralleled the level of Met reduction Fig. 10B, showing that reduction of Met expression can affect Met-dependent cell scattering without affecting cell viability or proliferation. Thus, si-dMet-Ad5^{197}-infected MDCK cells scattered the most and expressed th highest level Met, whereas the si-dMet-Ad5^{111}-infected cells scattered the least and expressed the lowest level of Met.

EXAMPLE 9

met RNAi Suppresses Signaling

HGF/SF binding to Met results in Met phosphorylation and activation of downstream pathways such as the phosphoinositide 3 kinase (PI3K)-Akt (Graziani, A *et al., J Biol Chem 266:*22087-22090, 1991) and the mitogen-activated protein (MAP) kinase pathways (Karihaloo, A *et al., J Biol Chem 276:*9166-9173, 2001). These pathways are essential for biological functions such as cell migration, proliferation, morphogenesis, and escape from apoptotic cell death.

After exposure to HGF/SF DA3 cells showed steady or increased level of Met phosphorylation for 2 h, followed by a gradual decrease (Fig. 8A) (Firon *et al., supra*). p44/42 MAPK and Akt were also rapidly phosphorylated 10 min after HGF/SF stimulation. In contrast, phosphorylation of p44/42 MAPK lasts longer in these cells. Endogenous phosphorylation of Met and p44/42 MAPK are observed in DA3 cells in the absence of HGF/SF stimulation (0 min) after serum starvation. This suggests that other signaling pathways are acting in these cells or Met activation is ligand-independent.

In non-infected control and mock virus (mU6-Ad5)-infected cells, Met was rapidly phosphorylated in response to HGF/SF. Increased phosphorylation of Akt and p44/42 MAPK was also observed. Although p44/42 MAPK phosphorylation was observed in si-mMet Ad5^{178}-infected cells, it was significantly suppressed compared with non-infected controls and mock infected cells. However, the phosphorylation of Met and Akt was almost completely abolished (Fig. 8B). Since Akt activation contributes to the stimulation of an anti-apoptotic pathway, the reduced level of phospho-Akt is consistent with increased susceptibility of si-mMet Ad5^{178}-infected DA3 cells to apoptotic cell death.

EXAMPLE 10

Met RNAi Inhibits Cell Invasion and Proliferation *In Vitro*

The influence of si-Met-Ad5 virus infection on Met-mediated *in vitro* invasion by DA3 mouse mammary adenocarcinoma cells was tested. Infected or mock-infected DA3 cells were plated in Matrigel® chambers and invasion assays were performed after treatment with HGF/SF. Without HGF/SF stimulation, the cells did not migrate and only a few cells were detected by Giemsa staining. In response to HGF/SF, however, non-infected control DA3 cells readily invaded Matrigel®;

approximately 80 cells wee detected per filter. In contrast, cells infected with met siRNA viruses showed markedly reduced invasion. Such activity was almost completely suppressed in the cells infected with si-mMet-Ad5^{178} which was correlated with interference of Met expression (not shown).

Likewise, si-hMet-Ad5^{221} dramatically suppressed invasion activity of human cancer cell lines. Cells of both PC-3 and SK-LMS-1 lines that are stably transfected with the human *hgf/sf* gene (SK-HGF) (Jeffers *et al.*, *supra*) displayed reduced invasion (not shown). PC-3 and SK-HGF cells are less dependent on extrinsic HGF/SF than are other human cancer cell lines. These results show that invasive activity was largely dependent on Met signaling. Interestingly, PC-3 cells show little or no proliferative or invasive response to HGF/SF and express high levels of Met (Nishimura, K *et al., Int J Urol 5:*276-281, 1998). However, the loss of Met revealed an inhibitory effect on invasive activity, suggesting that Met signaling is autonomous (no mutation found) perhaps by ligand independent activation.

The effect of RNAi on DA3 cell proliferation was tested. As measured by the MTT assay, the growth response after stimulation with HGF/SF was strongly suppressed by si-mMet-Ad5^{178} infection even at moi=10, and the suppressive effect peaked at moi=50. In contrast, mock mU6-Ad5 virus did not show any suppressive effect on cell growth at moi=10 to 50, and only mild suppression was observed at moi=100. These results suggested that met RNAi suppressed both invasion and proliferation, which are important for tumor cell progression.

EXAMPLE 11

met RNAi Suppresses Tumorigenesis

met RNAi suppressed the expression of Met protein and blocked scattering, proliferation, and invasion as well as downstream signaling *in vitro*. Experiments were done to test whether si-Met-Ad5 RNAi would also suppress tumorigenicity. The first study tested whether DA3 cells infected with si-mMet-Ad5^{178} which grow *in vitro* would also grow as tumors in BALB/c mice upon sc inoculation. Tumor growth was measured for 3 weeks, and a dramatic difference was observed. All mice (10/10) showed remarkable tumor growth when inoculated sc with uninfected "control" tumor cells. In contrast, tumor formation by DA3 cells infected with si-mMet-Ad5^{178} was almost completely suppressed ($p<0.001$ vs. uninfected control; $p<0.01$ vs. mU6-Ad5) (Fig. 9A).

si-mMet-Ad5 was tested for suppression of tumor formation *in vivo* (siRNA gene therapy). DA3 cells were inoculated subcutaneously in the flank of BALB/c mice, and then si-mMet-Ad5^{178} was injected directly into the tumor 3 and 7 d after tumor inoculation. The mice injected with si-mMet-Ad5^{178} virus showed a statistically significant reduction in the tumor size ($p<0.05$ to 0.01), whereas there was no remarkable difference in the tumor size between PBS control and mock virus (mU6-Ad5)-treated mice (Fig. 9B). This result supports the use RNAi for cancer treatment.

DISCUSSION OF EXAMPLES 1-11

RNAi is a potent tool for silencing the function of specific genes (Elbashir *et al.*, *supra*). The present Examples involved production of siRNA adenoviruses that carry target sequences against either mouse or human Met. The different adenoviral constructs showed different, reproducible patterns of suppression of Met protein expression (see Fig. 1). For instance, the rank order of Met suppression in mouse cell lines (M114 and DA3) was si-mMet-Ad5^{178} > si-mMet-Ad5^{110}, si–mMet-Ad5^{57} > si-mMet-Ad5^{60} (see Fig. 1A and 5C). In human cell lines (DBTRG, and PC-3), the rank order was si-hMet-Ad5^{221} > si-hMet-Ad5^{62} > si-hMet-Ad5^{16} (see Fig. 1B and 1C). si-hMet-Ad5^{221}, which was the most potent in human cell lines, did not affect Met expression in mouse DA3 cells, whereas si-mMet-Ad5^{178} produced a dramatic reduction in the Met protein level in those cells (see Fig. 3A). This effect was confirmed by evaluating mRNA levels (see Fig. 3B). These results show that the siRNA constructs of the present invention mediate RNAi effects in a sequence-specific manner.

The effective duration of RNA interference was examined. According to a previous report, silencing in dividing cells lasts only 3 to 7 d, presumably because of dilution of siRNA secondary to cell division (Song, E *et al.*, *J Virol 77:*7174-7181, 2003). Here, the greatest effects were observed after the first cell passage (day 6–7 after infection); RNAi was still evident after passage 2 (see Fig. 5). This means that RNAi lasts at least 9-10 d after siRNA adenovirus infection. Because these adenoviral constructs include a U6 promoter–based RNA transcription system, there should be more siRNA molecules per cell compared with cells transfected directly with siRNA molecules. After si-mMet-Ad5 infection, RNAi was induced swiftly, and close to a 50% reduction in Met expression was observed by 24 h (see Fig. 5C); the effect of RNAi increased gradually by 72 h. This suggested that short hairpin RNA can be transcribed efficiently after the siRNA adenovirus genes are established in the cytoplasm.

The efficiency of Met reduction by si-Met-Ad5 varied among the cell lines. DBTRG, PC-3, and MKN45 cells (see Fig. 1) responded better than did SK-LMS-1, and the suppression of Met expression in DA3 cells was more robust than that in M114 cells (see Fig. 1 and 5). Entry of adenovirus requires two receptors: a primary receptor known as the Coxsackievirus/adenovirus receptor (CAR) for attachment, and secondary receptors such as the $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins for internalization (Nemerow, GR, *Virology 274:*1-4, 2000). Recent reports showed that loss of CAR expression was a major limiting factor in adenovirus gene therapy (Pearson, AS *et al.*, *Clin Canc Res 5:*4208–4213, 1999). Since the cell lines of epithelial origin (DBTRG, PC-3, MKN45, and DA3) are considered to express higher levels of CAR than the non-epithelial lines (SK-LMS-1 and M114), the infectivity might explain the difference in Met reduction.

Abrogation of HGF/SF-Met signaling to suppress the Met-dependent malignant phenotype has been achieved by several different approaches. In one, a dominant-negative (DN) form of Met reduced

in vitro motility and invasiveness as well as the *in vivo* tumorigenic and metastatic potential, of DA3 cells (Firon *et al.*, *supra*)

Although the molecular mechanism of the DN-Met effect is not entirely clear, dimerization of the DN-Met receptor with the wild-type receptor is believed to interfere with HGF/SF-induced Met signaling. In the context of Met signaling, however, direct molecular targeting of the Met protein would be a more straightforward and robust way to test the mechanism. Abounader *et al.* (2002, *supra*) designed a U1snRNA/ribozyme for targeting Met and reported that it reversed the malignancy of glioma cells, inhibited the growth and angiogenesis, and promoted apoptosis. Targeted gene expression was inhibited effectively measured as mRNA and protein levels of 73–98% by using stable expression of U1snRNA/ribozyme. However, the efficiency of transient infection using an adenovirus system remained 75% reduction in mRNA and 50% reduction in Met protein. In contrast, the present si-Met-Ad5 system induced a more efficient reduction (confirmed by western blot analysis). Reduction in Met expression reached 62% in DBTRG cells, 68% in PC-3 cells, and 71% in MKN45 cells (see Fig. 1B, 1C, and 1D). In the more sensitive DA3 cell line, the reduction was between 85% and nearly 100% (see Fig. 3A and 5C). Despite a variation in the susceptibility to si-Met-Ad5 viruses among the cell lines analyzed, the present RNAi system provides a higher certainty of Met reduction than do other known methods.

The RNAi effect mediated by si-Met-Ad5 not only by suppressed invasion and proliferation, but also promoted cell death. Despite the fact that Met expression levels did not necessarily correlate with susceptibility to apoptosis, a reduction in Met protein triggered more cell death in cancer cell lines such as DA3 and MKN45, and in several other cell lines (to a lesser extent) (see Fig. 6 and 7, and Table 3). In contrast, M114 cells after si-Met-Ad5 infection underwent morphological changes without frank apoptosis (see Table 3), suggesting that Met expression is more important for survival of cancer cells than normal cells such as fibroblasts. Upon HGF/SF stimulation, the Met receptor is phosphorylated, followed by the recruitment of a group of signaling molecules and/or adaptor proteins to its cytoplasmic domain and multiple docking sites (Zhang, YW *et al., J Cell Biochem 88:*408-417, 2003). This leads to activation of several different signaling cascades that form a unique network in various types of outward responses; such as cell proliferation, cell migration, cell invasion, angiogenesis, and metastasis.

After infection with si-mMet-Ad5, DA3 cells showed remarkable suppression of scattering (see Fig. 4B), invasion, and proliferation, and similar results were observed in human cell lines (PC-3 and SK-HGF). Marked suppression of the phosphorylation of Met and of downstream molecules (Akt and p44/42 MAPK) was observed in si-mMet-Ad5-infected DA3 cells (but not in mock–infected cells). See Fig. 8B. These findings suggests that all the phenotypic changes are effected by suppression of the phosphorylation of Met and downstream molecules. HGF/SF signaling is known to stimulate the Akt pathway and to protect cancer cells from death (Bowers, DC *et al., Canc Res 60:*4277-4283, 2000).

HGF/Met can protect cells from apoptosis through both PI3-kinase/Akt and, to a lesser extent, MAPK pathways (Xiao *et al.*, *supra*). Actually, the present results with DA3 cells infected with si-mMet–Ad5[178] showed a very low Akt response while generally maintaining a MAPK signaling pathway (see Fig. 8B). This suggests the importance of the Akt pathway for viability of DA3 cells.

Since overexpression or active mutation of the Met protein is involved in a wide spectrum of solid tumors, Met is considered one of the key targets for cancer gene therapy (Ma, PC *et al., Cancer Metastasis Rev 22:*309-325, 2003. Recently, *in vivo* treatment approaches that target the HGF/SF–Met signaling were reported by several groups. One approach used the NK4 gene that acts competitively with HGF/SF (Maemondo, M *et al., Mol Ther 5:*177–185, 2002). However, this therapy is limited to tumor cells in which Met signaling is ligand-dependent. Other reports disclosed DN- Met (Furge *et al.*, *supra*) or Met ribozyme (Herynk, M.H *et al., Canc Res 63:*2990-2996, 2003) as targeting tools. Although these reports showed statistically significant suppression of tumor growth, there continues to be a need for improved genetic tools for more efficient anti-cancer activity.

This document provides the first disclosure that c-*met* siRNA adenovirus can effectively suppress Met expression and a wide variety of tumor functions, and that these effects could be manifest both *in vitro* (see Fig. 2, 4, 6 and 7) and *in vivo* (see Fig. 9).

It was concluded that the si-Met-Ad5 adenovirus constructs provide a powerful tool for the analysis of the HGF/SF-Met signaling pathway, as well as cancer therapy, preferably in the form of cancer gene (or nucleic acid) therapy.

All the references cited above are incorporated herein by reference in their entirety, whether specifically incorporated or not.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.